EVALUATION OF NEAR INFRARED REFLECTANCE (NIR) SPECTROSCOPY TO DETERMINE THE NUTRIENT COMPOSITION OF RAW MATERIALS AND COMPOUND OSTRICH FEEDS

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EVALUATION OF NEAR INFRARED REFLECTANCE (NIR) SPECTROSCOPY TO DETERMINE THE NUTRIENT COMPOSITION OF RAW MATERIALS AND COMPOUND OSTRICH FEEDS

by

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DECLARATION

I, Etheresia Swart, student no: 212472534, hereby declare that the thesis for MAGISTER TECHNOLOGIAE: AGRICULTURE to be awarded is my own work and that it has not previously been submitted for assessment or completion of any postgraduate qualification to another University or for another qualification.

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E Swart

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NOTE

This thesis was written according to the style and language used by the South African Journal of Animal Science. This thesis represents a compilation of chapters, each written in the form of a publishable article. Each chapter therefore consists of an abstract, introduction, materials and methods, results and discussion, conclusion and references. Each chapter must be seen as a unit on its own, but together it forms a whole. Because chapters are written in article format, it is unavoidable that some information contained in the literature review is repeated to some degree in the respective chapters. This is to provide context for the particular article when read on its own. To date, one scientific publication and a number of conference poster presentations have resulted from this study, as indicated below:

Publications

Swart, E., Brand, T.S. & Engelbrecht, J., 2012. The use of near infrared spectroscopy (NIRS) to predict the chemical composition of feed samples used in ostrich total mixed rations. South African Journal of Animal Science 2012, 42 (Issue 5, Supplement 1).

Conference posters

Swart, E., Brand, T.S. & Manley, M., 2010. The use of near infrared spectroscopy (NIRS) to predict the chemical composition of ostrich feed samples - 28th South African Society of Agricultural Technologists Conference. Mabalingwe, Bela Bela. 7 - 9 September.

Swart, E., Brand, T.S. & Manley, M., 2011. The use of near infrared spectroscopy (NIRS) to predict the chemical composition of ostrich feed samples - 44th South African Society of Animal Science Conference, Stellenbosch University, Stellenbosch. 11 -14 July.

Swart, E. & Brand, T.S., 2011. The prediction of oleic and linoleic acid content of sunflower seeds using near-infrared reflectance spectroscopy (NIRS) - 29th Congress of the

South African Society of Agricultural Technologists. Phantom View River Resort, Knysna. 6 - 9 September.

Swart, E. & Brand, T.S., 2012. The prediction of oleic and linoleic acid content of sunflower seeds using near-infrared reflectance spectroscopy (NIRS) - 45th South African Society of Animal Science Conference. East London. 9 - 12 July.

Swart, E., Brand, T.S., Engelbrecht, J.A.A., Lehmann-Maritz, M. & Terblanche, S., 2013. The use of near infrared spectroscopy (NIRS) to predict the chemical composition of canola seed (*Brassica napus* L.) - 46th South African Society of Animal Science Conference. Bloemfontein. 23 - 26 June.

Swart, E., Brand, T.S., Engelbrecht, J.A.A. & Lehmann-Maritz, M., 2013. Prediction of chemical composition in milled canola seed (*Brassica napus* L.) by near infrared reflectance spectroscopy (NIRS) - 31st Congress of the South African Society of Agricultural Technologists. Kedar Country Lodge, Rustenburg. 17 - 20 September.

Swart, E., Brand, T.S., Engelbrecht, J.A.A. & Lehmann-Maritz, M., 2014. The use of near infrared reflectance spectroscopy (NIRS) to predict the amino acid content of ostrich feeds - 32nd Congress of the South African Society of Agricultural Technologists. Arniston Spa Hotel, Arniston. 2 - 5 September.

Swart, E., Brand, T.S., Lehmann-Maritz, M. & Engelbrecht, J.A., 2015. Near infrared reflectance spectroscopy (NIRS) as a rapid method for the prediction of amino acids in mixed compound ostrich feeds - 48th South African Society of Animal Science Conference. Kwa-Zulu Natal. 21 - 23 September.

ABSTRACT

The chemical analysis of feed samples can be time consuming and expensive. The use of near infrared reflectance (NIR) spectroscopy was evaluated in a range of studies as a rapid technique to predict the chemical constituents in feedstuffs and compound ostrich feeds. The prediction of accurate results by NIR spectroscopy relies heavily upon obtaining a calibration set which represents the variation in the main population, accurate laboratory analyses and the application of the best mathematical procedures.

This research project was designed to meet five objectives:

The first objective was to determine the feasibility of using near infrared reflectance (NIR) spectroscopy to predict dry matter, ash, crude protein, crude fibre, oil content, and fatty acids such as palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2) in sunflower seed meal.

The second objective was to develop calibration models to predict the dry matter, crude protein and oil content in milled canola seed, compared to whole canola seeds.

The third objective was to investigate the feasibility of using NIR spectroscopy to predict the dry matter, ash, crude protein, crude fibre and oil content in milled lupin seeds, compared to whole lupin seeds.

The fourth objective was to describe the development of near infrared reflectance (NIR) spectroscopy calibration equations for the prediction of chemical composition and amino acid content from different populations of alfalfa hay (*Medicago sativa* L.).

The last objective was to determine the potential of NIR spectroscopy to predict the dry matter, ash, crude protein, crude fibre, ether extract, acid detergent fibre (ADF), neutral detergent fibre (NDF), calcium, phosphorus, *in vitro* organic matter digestibility (IVOMD) and amino acids such as lysine, methionine, threonine and arginine in compound ostrich feed samples.

The results of this study indicate that NIR spectroscopy calibrations in sunflower seed meal are only applicable in sunflower breeding programmes for a fast screening as it was not suitable for prediction purposes. Screening of sunflower seeds by NIR spectroscopy represents a rapid, simple and cost effective alternative that is a great utility for users who need to analyse a large number of samples.

Calibrations developed for crude protein and oil content in milled canola seeds proved to be better than calibrations for whole canola seeds. Although the results indicated

that calibrations were better for milled canola seeds, it indicated values that were typical of equations suitable for screening purposes to select samples for more detailed chemical analysis.

According to calibration statistics obtained for crude protein, crude fibre and oil content in whole lupin seeds, there is no need to grind the seeds to scan the meal as similarly accurate results were obtained by analysing whole seeds. Screening of whole lupin seeds by NIR spectroscopy represents a rapid, simple and cost effective alternative that may be of great utility for users who need to analyse a large number of samples with no sample preparation.

The calibration and validation statistics obtained in the study to test the potential of NIR spectroscopy to predict the chemical composition and amino acid contents in alfalfa hay, showed the accuracy was too low for routine analysis, although NIR spectroscopy could be used as a screening tool. Further research needs to be done to improve the accuracy of the NIR spectroscopy analysis, including more samples from different cultivars and years.

In the study to examine the possibility of using NIR spectroscopy to predict the chemical composition of compound ostrich feeds, the results indicated that NIR spectroscopy is a suitable tool for a rapid and reliable prediction of the crude protein, crude fibre, ether extract, IVOMD, ADF and NDF in compound ostrich feeds. Calibrations can be improved for amino acids if a larger sample pool is used to develop the calibrations.

These studies indicated that NIR spectroscopy can be a rapid and successful tool for the prediction of the nutritive value up to certain amino acid contents of feedstuffs and compound ostrich feeds.

ACRONYM GUIDE

ADF	Acid detergent fibre
AME	Apparent metabolisable energy
AOAC	Association of Official Analytical Chemists
Ca	Calcium
CP	Crude protein
CV	Coefficient of variability or variation
DM	Dry matter
EE	Ether extract
FAME	Fatty acid methyl esters
GC	Gas chromatograph
GE	Gross energy
HCl	Hydrochloric acid
HPLC	High pressure liquid chromatography
IVOMD	*In vitro* organic matter digestibility
MLR	Multiple linear regression
MPLS	Modified partial least square regression
MSC	Multiplicative scatter correction
n	Amount
N	Normality
NDF	Neutral detergent fibre
NFC	Non fibre carbohydrates
NIRS	Near infrared reflectance spectroscopy
Nm	Nanometers
NMR	Nuclear magnetic resonance
P	Phosphorus
PCA	Principal component analysis
PLS	Partial least square
RMSD	Root mean square deviation of differences
RMSEP	Root mean square error of prediction
RPD	Ratio of SEP to SD
rpm	Rotations per minute

SD	Standard deviation
SEC	Standard error of calibration
SECV	Standard error of cross validation
SEP	Standard error of prediction or standard error of performance
SET	Standard error of a single test or precision
SNV	Standard normal variate
TDN	Total digestible nutrients
TLC	Thin-layer chromatography
TTE	True test error

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CHAPTER 1

LITERATURE REVIEW

General introduction

The composition of diets for production animals is becoming increasingly important for several reasons. The feed industry, for example, requires rapid and accurate information regarding the nutritive value of feedstuffs, as such information is needed to negotiate the proper price for a feedstuff and to correctly include this feedstuff in a complete diet. Moreover, the nutritional requirement of the animal needs to be met with the lowest feed cost and such information is needed rapidly in order for routine evaluation of the feedstuffs to be possible (van Kempen, 1996). The intensive farming of livestock also requires constant monitoring of diet composition to ensure consistent levels of milk or meat production. Additionally, selection or breeding for nutritive value is now considered an important factor by major forage-plant breeders and there is often the need to test a very large number of lines (Deaville & Flinn, 2000). Although the traditional wet chemistry analysis does provide valuable information regarding feed quality, it is time consuming and expensive. Moreover, animal production is facing new challenges, including the need to formulate diets that not only offer the maximum differential between feed costs and product sales, but which also reduce the negative impact on the environment.

The nutritive value of animal feeds has traditionally been expressed in energy and protein values, obtained from wet chemical analysis and from metabolic trials using animals for which the feed is intended. Research has been done to develop equations to predict *in vivo* data based on chemical analysis. Although this approach is a reasonable compromise between simplicity and accuracy of prediction, it is relatively time consuming (Givens & Deaville, 1999), expensive, and can only be conducted at scientific institutes. Currently, many feed mills use near infrared reflectance (NIR) spectroscopy instruments to predict protein, moisture, fat and ash contents of feedstuffs to obtain the required information regarding nutritive quality of a diet and for quality control purposes. The NIR spectroscopy technology is used for monitoring the nutritional profile of a feedstuff which allows the detection of outliers that requires additional analyses and animal nutritionists can apply the information to reformulate diets (van Kempen, 1996).

NIR spectroscopy is a long-established, and now mature, technology. The first application of NIRS was developed by Norris & Hart (1965) by predicting the moisture content in grains and seeds with little sample preparation. The technology uses simple sample preparation methods such as drying and grinding. It is a very rapid technique as measurements can be done in seconds once the sample is prepared and is an inexpensive technique (Dryden, 2003). Since no reagents are required, the technique avoids the additional costs of organic and other chemical waste disposal, and there are few, if any, hazards associated with the technique, because no toxic or corrosive reagents are used (Mark *et al*., 2002).

The prediction of accurate results by NIR spectroscopy relies heavily upon obtaining a calibration set which represents the variation in the main population, accurate laboratory analyses and the application of the best mathematical procedures, although a large number of samples is needed to set up an accurate calibration (Aufrère *et al*., 1996).

The NIR spectroscopy technique is not only a rapid method, but also has other advantages over conventional chemical techniques, for instance, it is a physical, non-destructive method, which requires minimal or no sample preparation because samples can be presented directly to the instrument without any pre-treatment (González-Martín *et al*., 2006a; Pasquini, 2003). Moreover, in contrast to traditional chemical analysis, it is non-polluting, as no hazardous chemicals are required, and no waste products are produced, and can be carried out during a short period of time (Aufrère *et al*., 1996). A further advantage is that it can be performed regardless of the presentation of the feed (pellets, granules, grain, meal) and of the physiological stage of the animals (lactation, growth, maintenance, etc.) to which the feed is being fed (González-Martín *et al*., 2006a). Furthermore, it is a multi-analytical technique which allows for several chemical parameters to be predicted simultaneously and is simple to use and operate, once the NIR spectroscopy is calibrated (Smith & Flinn, 1991).

The main disadvantages are the need for high-precision and expensive spectroscopic instruments and it is dependent on time-consuming and labour-intensive calibration procedures and complicated data treatment. There can also be difficulties in the transfer of calibrations between instruments (Givens & Deaville, 1999; Smith & Flinn, 1991).

As conventional chemical techniques to determine the nutritive composition of feeds are expensive, time-consuming and sometimes hazardous, plant breeders, farmers and

animal nutritionists are in need of a reliable, precise, fast and cost-effective method to evaluate the nutritive value of pastures (Smith & Flinn, 1991). By making use of NIR spectroscopy, the results are made available in a matter of seconds. NIR spectroscopy is thus an invaluable tool to estimate the nutritive value of feeds (Givens & Deaville, 1999).

Study problem

In recent years, the livestock industry has had to cope with unpredictable crop yields, as well as an increased competition for raw materials from the bio-fuel industry. As a result, there has been an increase in the cost of feed ingredients and more industrial by-products have become available. At the same time, there are increasing demands for animal production to become more sustainable, for example, by decreasing excessive production while producing more food for a growing world population. To add to these problems, food prices have come under increasing pressure from the retail sector. These factors have all exerted pressure on the animal production sector to implement more professional and accurate practices (Graham *et al*., 2013).

As feed costs are responsible for 50 - 80% of the total variable costs of animal production, nutrition is an area of major concern. The main aim for nutritionists is to provide the animal with the correct amount of nutrients required for each production phase. Both surplus, as well as a deficit in nutrients, is translated into economic losses through higher feed costs or lower performance, respectively. Thus, it is important for the nutritionist and purchaser to have accurate information on the nutritional value of all available ingredients. Regular analysis of feed ingredients and feeds need to confirm that the nutrient content of feed is according to the formulation and should be a key part of a quality control system (Graham *et al*., 2013).

Nutritionists have traditionally used conventional chemical analysis to determine the nutrient composition of raw materials and feeds. Unfortunately, most of these techniques are time-consuming and expensive, which limit the amount of samples that can be analysed and creates a delay between sampling and the production of results (Graham *et al*., 2013).

In recent years, the demand has increased for the analysis of nutrient composition of feeds and quality control has been met by adaptation of various techniques. One of these techniques that were found to be suitable for the measurement of a large amount of constituents, is based on the interaction of the sample with NIR spectroscopy radiation. A

beam transmitted or reflected by a sample contains information on the properties of the sample. A large number of food components have absorption peaks in the near infrared spectral region of 1100 - 2500 nm; therefore, this region is particularly useful for the determination of the composition of feed products. A beam transmitted or reflected by the sample is measured through the near infrared spectral range and the resulting spectra or their transformed curves are used to derive the composition of the sample (Kaffka *et al*., 1982).

The development of the NIR spectroscopy technology during the last two decades has opened new perspectives to meet the demands to evaluate the quantitative and qualitative components of a wide range of organic materials (de Boever *et al*., 1995). NIR spectroscopy has other advantages over chemical techniques, in that it is a non-destructive method which requires only a very small sample. Several analyses can be carried out simultaneously once calibrations are established (Smith & Flinn, 1991).

NIR spectroscopy technology, which is based on analysis of animal feeds and feed ingredients, can rapidly and economically provide objective nutritional information on the diet of livestock, their likely productivity of ingredients, and processes for animal feed manufacturing. It allows for easy and comprehensive application of established nutritional science to the nutrition and management of livestock. This in turn improves the efficiency and productivity of livestock for food. Because a wide range of nutritional analyses can be conducted simultaneously with one instrument and a desktop computer, NIR spectroscopy can greatly reduce the capital investment, training and operational costs required for nutritional analysis and decision support (Dixon & Coates, 2010).

Background

About near infrared reflectance spectroscopy

NIR spectroscopy was introduced as a potential analytical tool for agricultural products by Karl Norris, an instrumentation engineer for USDA-ARS in Beltsville, Maryland, USA. The NIR spectroscopy technique has been used since 1965 (Norris & Hart, 1965) to analyse chemical constituents, such as crude protein and other organic compounds, in animal feeds, and a huge advantage is that it is a rapid, non-destructive and non-polluting technique. However, NIR spectral information cannot be used to determine the concentrations of constituents directly, because of the way in which near infrared radiation passes into, through and is reflected from the sample. The concentration

of the constituents that needs to be measured, has to be predicted by developing equations based on reference data. The chemical constituents or other characteristics of the sample are predicted from calibration equations, which are derived from correlations that have been developed between spectra and reference data. It is also important to apply appropriate mathematical techniques (e.g. smoothing and derivatisation) to the NIR spectroscopy data and to ensure the samples that are analysed are uniform in particle size and water content (Dryden, 2003).

NIR spectroscopy predicts crude protein contents with great precision when calibration equations are properly developed. Other constituents are predicted with less accuracy, although the standard errors of prediction are similar to the standard errors of duplicate laboratory determinations. NIR spectroscopy predictions are used with great precision with both concentrate and forage foods. NIR spectroscopy information is derived from the interactions of near infrared radiation with chemical bonds between non-mineral elements and therefore does not always predict feed mineral contents with great precision (Givens & Deaville, 1999).

Different models and several trademarks of NIR spectroscopy instruments are available for different needs. Suggested criteria to decide which one to choose, should include the range of intended applications and the software available for the specific applications. For poultry and animal feeds in general, a relatively large number of ingredients and wide variety of applications for different constituents is needed. For this reason the hardware and software should be carefully selected. The range of wavelengths frequently used in agricultural applications is 1100 – 2500 nm (Ruiz, 2001).

Theory and operation

The NIR spectroscopy technique is based on the absorption of electro-magnetic radiation in the near infrared region (1100 – 2500 nm) by specific chemical bonds in the sample. A computerized optical device is used to collect this information for a set of samples of known composition. The derived relationship is then used to analyse similar samples of unknown composition (Smith & Flinn, 1991). Infrared is the region of the electromagnetic spectrum located after the visible region in the direction of longer wavelengths. Near infrared owns its name for being the “near” section of the infrared region to the visible region. Spectroscopy specialists divide the infrared region into near,

middle and far infrared. For practical purposes, near infrared comprise wavelengths between 800 and 2500 nm (Ruiz, 2001).

NIR spectroscopy technology is based on the absorbance of light energy of a given frequency by molecules, having a permanent dipole, which vibrate at the same frequency. The difference between the incident light and light reflected from the surface of the sample is analogous to the familiar Beer-Lambert concept of absorbance/transmittance (Panford *et al*., 1988). The primary advantage of the NIR region is that absorbances are lower than in neighbouring regions and generally obey the Beer-Lambert law, i.e., absorbances increase linearly with concentration. This is because NIR absorptions are generally 10-100 times weaker in intensity than the fundamental mid-infrared absorption bands. The weakness of the absorptions is a benefit, providing direct analysis of samples without dilution or the requirement of short optical path lengths or dispersion in non-absorbing matrices used in traditional sampling techniques in UV/VIS and mid-infrared spectroscopies (Williams, 2012).

When a sample is irradiated with light, according to energy conservation law, fractions are reflected, transmitted, and absorbed, all summing to 1.0. The proportions depend on the light wavelength and sample properties (composition and thickness among others). Beer's law, well-known in molecular spectroscopy, defines the correlation of the concentration of constituents with its absorbance at specific wavelengths. Beer's law is not directly applicable in NIR spectroscopy because of several restrictive assumptions: no correlation between multiple absorbers, homogenous samples, negligible light scattering, and constant path length. Notwithstanding this, Beer's law implication is still held by NIR spectroscopy analysis (Agelet & Hurburgh, Jr., 2010).

NIR spectra are formed of overtones and combination bands. Overtones are electron excitations to higher energy levels, which occur at multiples of the mid-infrared (MIR) fundamental frequencies. The study of spectroscopy involves the interaction between electromagnetic radiation and matter as a function of wavelength. Electromagnetic radiation is absorbed by chemical bonds when the energy of a light photon is equal to the energy difference between two vibrational and rotational states of a chemical bond. Energy and wavelength are equivalent to each other and can be converted from the one to the other. Thus, the wavelengths of absorbed radiation are unique for each molecule; intensity of absorption is proportional to the concentration of molecules and therefore can be interpreted to understand the composition of a substance. The initial absorptions by

organic molecules are in the infrared region. They are the fundamentals that result in narrow absorption peaks and can be directly interpreted to determine the composition of a substance (Walker & Tolleson, 2010).

The NIR spectra are treated mathematically to ‘extract' the information from the sample. All biological substances contain thousands of C-H, O-H and N-H molecular bonds. Therefore, the exposure to near infrared radiation of biological samples, such as feed ingredients, results in a complex spectrum which contains qualitative and quantitative information about the physical and chemical composition of that sample. Moreover, every biological substance has a unique NIR spectrum. If two biological samples have the exact same spectrum, it can be assumed that they have the exact physical and chemical composition. If spectra are different, then the samples are different either physically or chemically or both (Ruiz, 2001).

The NIR spectroscopy technique is applied to organic compound bonds which are the primary constituents of the organic molecules of which feeds consist. The bonds are rich in O-H bonds (such as moisture, carbohydrates and fat), C-H bonds (such as organic compounds, petroleum derivatives) and N-H bonds (such as proteins, amino acids). The absorption of NIR radiation by organic molecules is due to overtone and combination bands primarily of O-H, C-H, N-H and C=O groups whose fundamental molecular stretching and bonding absorb in the mid-infrared region (Williams, 2012).

Peaks in the log (1/R) spectrum represent the harmonics, overtones and combinations which arise from the primary absorption in the mid-infrared spectrum (Coleman & Murray, 1993). Overtones represent whole integer multiples of the much stronger fundamental absorption frequencies found in the mid-infrared region (2500 - 50000 nm). Combinations arise from the sharing of NIR spectroscopy energy between two or more fundamental absorptions. Special sample preparation is not necessary when using NIR spectroscopy, because NIR radiation has more energy than the mid-infrared region where fundamentals are located and longer path lengths are possible. However, overtones and combinations create complex NIR spectra with broad absorption bands that are composed of multiple narrow, overlapping absorptions. NIR spectra are much more complex than they appear and were not useful until the advent of high-speed computers and multivariate algorithms to convert complex spectra to useful information. Even so, the NIR spectrum contains a tremendous amount of information and should provide some

fundamental understanding of the digestion process (Walker & Tolleson, 2010; Coleman & Murray, 1993).

There are no necessary chemical or physical relationships between the constituent under consideration and the wavelengths which may be selected to predict it. The way NIR spectroscopy instruments operate is by statistically correlating NIR signals at several wavelengths with the compound intended to be predicted. Reflectance in the near infrared spectrum represents the chemical structure of the sample (Purnomoadi *et al*., 1996).

Transmittance vs. reflectance

NIR spectra can be measured as transmittance or reflectance. The reflectance mode is based on reflecting near infrared radiation from the surface of the sample to the detector and by transmittance the near infrared radiation passes through the sample. The samples need to be milled to a uniform surface when measurement is taking place in reflectance mode and for transmittance very little or no sample preparation is necessary. For that reason transmittance is a faster method and more reproducible than reflectance, but transmittance is less sensitive than reflectance (Ruiz, 2001).

Light energy directed at an uneven or granular surface is either specularly or diffusely reflected. Specular reflectance is reflected directly from the surface and contains no information relative to chemical bonds. Other portions of the spectra are absorbed by the molecular bonds in the sample before the remaining energy is reflected back to the detector. The radiation that enters the sample and is reflected back is termed “diffuse reflection” because it becomes diffused by random reflections, refractions and scatter at further interfaces inside the sample. This reflected energy is affected by particle size of the sample, and the observed spectrum contains information about both the chemical and physical nature of the sample. The nature of diffuse reflectance allows multiple constituents and physical properties to be determined from a single diffusely reflected spectrum (Walker & Tolleson, 2010).

Advantages and disadvantages

There are various advantages to using NIR spectroscopy instruments; for instance, the technique is capable of providing rapid analysis which is nearly instantaneous in measurement. Furthermore, modern NIR spectroscopy instruments can perform multiple scans and average them in less than a minute and sometimes in less than a second

(Mark, 2012). The operation of NIR spectroscopy instruments are also straightforward and completely safe to operate and avoid the need for the use of chemical reagents that are harsh, toxic, corrosive and expensive. Moreover, since no reagents are required, no extra expenses are involved for solvent waste disposal and there are minimal, if any, hazards associated with NIR spectroscopy techniques. NIR spectroscopy methods can also reduce the costs of testing. For routine chemical analysis, the cost of NIR spectroscopy was calculated at about one-third of the cost of traditional wet chemistry analysis. Additionally, the analysis of constituents requires little or no sample preparation and several more analysis per day can be performed, as no associated reagent preparation steps or sample preparation steps are required (Ruiz, 2001; Mark *et al*., 2002). The NIR spectroscopy method also offers wide flexibility in sample presentation, as it is capable of analysing large and inhomogeneous samples which reduces sampling error (Williams, 2012; Mark, 2012).

The performance of reference analysis is of utmost importance, since the accuracy of the calibration, and in particular its subsequent monitoring, depends largely on that of the reference testing. The reference data must be paired with the correct optical data, as it can be one of the most consistent reasons for outliers (Williams, 2001). NIR spectroscopy predictions provide analysis with the same accuracy and reproducibility as traditional wet chemistry analysis, but are often limited by the accuracy of the method used to provide values with which to compare the NIR spectra. However, it does not require any drainage or fume exhausts, and the installation is uncomplicated. Routine analysis can be done by staff with minimal training or technical expertise (Dixon & Coates, 2010). Samples are analysed rapidly without destroying them which makes it suitable to analyse live material which can still be used for other purposes, rather than being discarded. Multiple components of each sample can also be determined from a single measurement of a sample's spectrum. Both the composition and functional properties can be measured and pure compounds can positively be identified if a library of compounds is developed. Furthermore, no consumables are required for NIR spectroscopy instruments, except lamps which need to be maintained. Samples can be analysed in the laboratory, in-line or in the field with portable instruments (Williams, 2012; Murray, 2010). To obtain the most accurate predictions by NIR spectroscopy, calibration models need to be developed on single feedstuffs. This requirement can only be met if a large number of samples are included in the database to include all possible variations (van Kempen, 1996).

Disadvantages of using the NIR spectroscopy technique are that the initial capital investment of the NIR spectroscopy instrument is high and software and the development of calibrations is time consuming (Ruiz, 2001). Moreover, sampling error can be caused by the lack of homogeneity in the materials being sampled. The variation differences between the samples are often the effective lower limit on the standard error of calibration (SEC) in an NIR spectroscopy calibration (Hruschka, 2001). Another inconvenience in NIR spectroscopy technology is the need to calibrate the instrument for each commodity and component of each type of sample to be analysed (Williams, 2001).

One of the biggest drawbacks of NIR spectroscopy technology is that it is not a stand-alone technology. In order to gain the benefits of NIR spectroscopy technology, it is necessary to perform fairly complicated and intricate calibration procedures and considerable technical skills are required to develop and maintain calibration equations. A high level of technical expertise, knowledge and skills is needed to develop new calibrations or adapt existing calibration equations. Considerable training and experience are usually required to become an expert in chemometrics and the specialised software packages required. Calibration equations are usually quite specific for the product or material being measured; thus, they will usually need to be developed for regional situations. This usually requires analysis of both NIR spectroscopy and conventional chemistry of many hundreds of samples to be used to develop the calibration before analysis of unknown samples can commence (Dixon & Coates, 2010).

Furthermore, the calibration procedure involves the measurement of the spectra of a large number of samples, followed by complex calculations that allow the computer program to determine the relationship between the spectra of the samples and their compositions. The calibration procedure is time consuming and very costly (Mark, 2012). A reduced set of samples must still be measured by wet chemistry techniques. The measurement of samples outside the range of the calibration samples will be invalid. Small calibration sample sizes can lead to overconfidence (Murray, 2010).

Moreover, the accuracy of NIR spectroscopy is dependent upon the accuracy and precision of the reference methods, although the predictions can be more reproducible than the reference method. Separate calibrations are required for each constituent or functionality parameter. To ensure that calibrations remain reliable, the accuracy of calibrations should be monitored by periodical analysis of some of the samples being predicted by the reference method. It may be necessary to update calibrations several

times during the initial phases of use to incorporate samples representing new variances not encountered previously, until the calibrations have become highly robust (Williams, 2012).

Sample requirements for development of calibrations

The database or library of spectra that is needed to develop calibrations should cover a wide range of variability for the constituents (Ruiz, 2001). A minimum of 50 samples, but preferably 200 or more samples that have been accurately analysed for the constituents or parameters of interest by traditional chemistry methods is needed. Samples should represent the full range of concentrations of the constituents of interest that need to be predicted in future samples by the calibration. Ideally, the highest values should be at least twice and preferably ten times the lowest values. Samples should represent the range of physical and chemical compositions anticipated in future samples to be predicted by the calibration. Samples should represent a range of environmental conditions, such as temperature. Samples should be chemically and physically unchanged between the time the constituents are scanned by NIR spectroscopy and analysed by conventional methods. Samples should be physically uniformly mixed so that the aliquots analysed by conventional method and those scanned by NIR spectroscopy are truly representative of one another. Ideally, the actual sample analysed by the conventional methods and scanned by NIR spectroscopy should be the same, but is not feasible in practice (Williams, 2012). More robust calibrations may use a few hundred samples, for instance, instrument built-in calibrations for grain analysis. Calibrations of homogeneous mixtures may require smaller calibration sets than agriculture samples of high compositional complexity and heterogeneity, such as whole grains or forages (Agelet & Hurburgh, Jr., 2010).

Accuracy in the determination of laboratory reference values for use in NIR spectroscopy calibration development has been accepted as a critical component of useful NIR spectroscopy technology. Conventional wisdom dictates that the accuracy of NIR spectroscopy predictions can only be as good as the laboratory reference values used for calibration (Coates, 2002) and comprehensive sample selection is essential, but the samples are not always available. A large amount of samples for calibration development is necessary, but is time consuming and expensive (Martens & Naes, 2001). The NIR spectroscopy technique is strictly correlative; therefore, the choice of calibration samples is critically important. The calibration samples should include all the variability in composition

(range of constituent values), particle size, sample treatment, etc., that might be encountered in any sample in practice to be measured (Kaffka *et al*., 1982).

Calibration procedure and model validation

NIR spectroscopy analysis depends on the development of mathematical relationships (calibration equations) between absorbances at various wavelengths in the NIR region and composition of reference samples determined by conventional procedures, such as wet chemistry. The NIR spectroscopy absorbance spectra of unknown samples are then used with these calibration equations to estimate constituents and functional properties (Dixon & Coates, 2010). Calibrations are statistical operations performed on spectral data to obtain an optimal statistical relationship between the spectral data and the reference data. The calibrations are the basis of predicting future, unknown samples in a rapid manner. In common with conventional spectrophotometric analysis, the calibration provides a simple linear regression relationship between spectral data and the concentration of a constituent (Williams, 2012).

The calibration set should be selected in such a way that it covers the full variation in the sample, including differences in physical properties. Because the calibration range should be wider than the specified range, samples without amounts of the quantified substance out of specification should be included (Anonymous, 2003).

The samples that are used for the calibration must represent the sample variation to be used for analysing the future samples. The range of the values of the constituents in the calibration sample set must equal or exceed the ranges of those constituents to be encountered in the future, when the analyser is put to routine use (Mark, 2012).

NIR spectroscopy instruments determine protein and other constituents by measuring log (1/R) values that must be correlated to the values of the constituents as determined by wet chemistry analysis, which are termed a reference or standard method. To establish this correlation by using a set of samples of known composition, is termed calibration of an NIR spectroscopy method; whereas using the correlation to determine the amount of a constituent in a new sample is called an NIR spectroscopy determination or prediction. The correlation between the log (1/R) values and the reference method values, is expressed as an approximation and always involves some form of regression equation. The amount of radiation reflected from the sample is quantified as the reflectance (R) of the sample. The value is usually expressed as log (1/R), which gives higher values at

higher levels of absorbance (i.e. lower reflectance). There is an almost linear relationship between log (1/R) and the concentration of an absorbing component. The regression equation has regression constants (the Y-intercept and regression coefficients), independent variables, and one dependent variable (the reference method value). The independent variables are mathematical combinations of log (1/R) values at various wavelengths. These combinations can be so complicated that they are better thought of as a series of steps, thus the term data treatment can be used to mean any mathematical process that combines log (1/R) values with independent variables for use in a regression equation. Developing a calibration model involves testing different data treatments, data treatment constants, or sets of wavelengths. Calibration means finding the regression constants that go into the approximation once the form of the approximation, the data treatment constants, and the wavelengths have been decided upon (Hruschka, 2001).

In order to estimate the functionality of the calibration model, predictions of an independent validation set must be evaluated in a process called validation (Malm *et al*., 2012). An adequate validation of the calibration model is a crucial step to determine the suitability of the model to predict new samples, which is the whole purpose of developing NIR spectroscopy calibrations. Ideally, the best validation should be done with a number of samples that covers a range of samples which were not previously used for development of the calibration equation and which can be encountered in future predictions. Since independent validation may not always be possible, cross-validation can provide a basic assessment regarding calibration performance. The general idea of the method is to keep a single sample (full cross-validation) or a group of samples (k-fold cross-validation) apart and develop a calibration with the remaining samples. The developed calibration is validated with the excluded samples and the prediction values are recorded. This procedure is consecutively done until all samples have been predicted once. The final calibration model is not tested, but rather several sub-models developed with calibration data subsets. Any statistic reported from cross-validation cannot be directly compared or interpreted the same way as statistics from a real validation of the final model with new samples. Reporting cross-validation statistics is preferred over reporting calibration results alone (Agelet & Hurburgh, Jr., 2010).

In order to develop calibration equations, the process must consist of the following steps: (i) collection of spectral data; (ii) pre-processing of spectral data to eliminate noises and baseline shift from the instrument and background; (iii) building a calibration equation

by using a set of samples with known analysed concentrations obtained by suitable reference methods; and (iv) validation of the models by making use of another independent set of samples not used in the calibration set (Cen & He, 2007).

The principal steps to follow during the development of a quantitative model based on NIR spectroscopy, is illustrated in Figure 1.

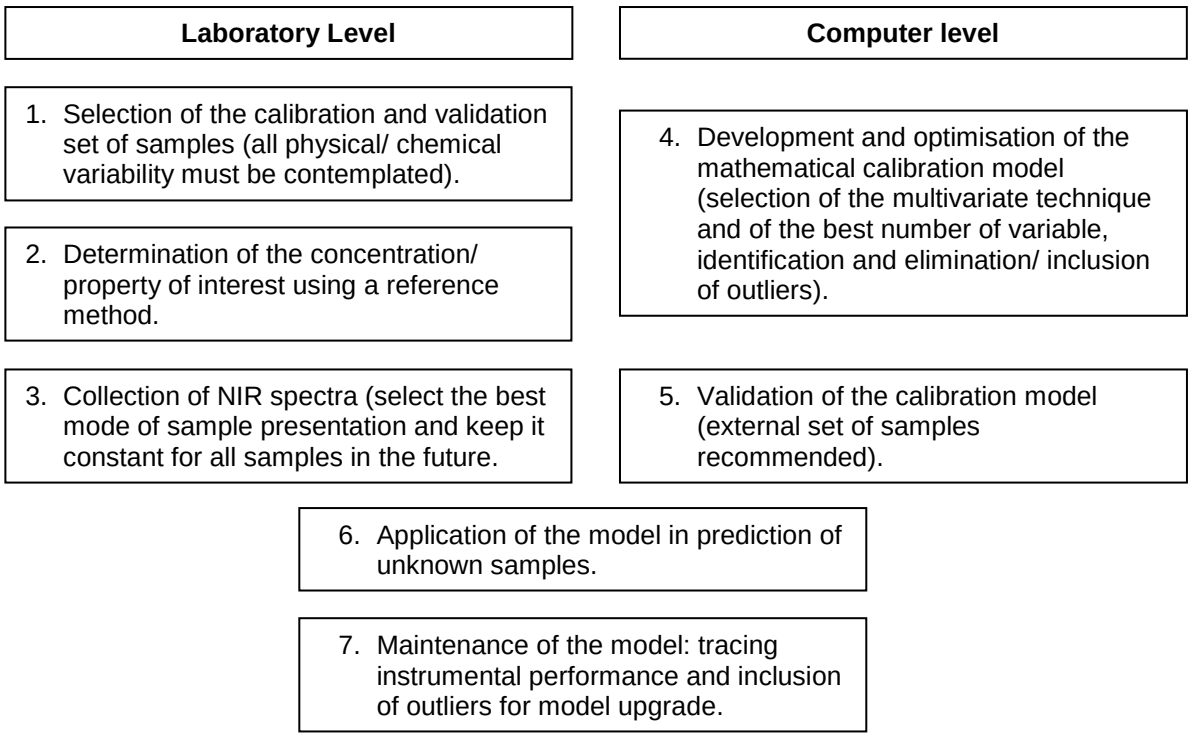

Figure 1 Principal steps in the development, evaluation, use and maintenance of a quantitative model based on NIR spectroscopy (Pasquini, 2003)

The NIR spectroscopy standard was developed from 2007 to 2010 by more than 20 NIR spectroscopy specialists and users, and was published in 2010 as International Organization for Standardization (ISO12099) - Guidelines for the application of near infrared spectroscopy (ISO12099, 2010). ISO12099 is a general standard that focuses on the validation of calibration models with independent test sets. The International Standard provides guidelines for determination of nutrient contents such as moisture, fat, crude protein, starch and crude fibre by NIR spectroscopy, as well as parameters such as the digestibility in animal feeding stuffs, cereals and milled cereal products. The standard

includes any type of instrument which is based on diffuse reflectance or transmittance measurement covering the near infrared wavelength region of 770 - 2500 nm. The optical principle may be dispersive (e.g. grating monochromators); interferometric or non-thermal (e.g. light emitting diodes, laser diodes and lasers). The instrument should be provided with a diagnostic test system for testing photometric noise and reproducibility, wavelength/wave-number accuracy, and wavelength/wave-number precision (for scanning spectrophotometers) (Möller, 2013).

In order to establish successful validations, it is important to have a wide range of representative samples which includes the following variations: (i) combinations and composition ranges of minimum and maximum sample components, (ii) seasonal, geographic and genetics effects on forages, feed raw materials and cereals, (iii) processing techniques and conditions, (iv) storage conditions, (v) sample and instrument temperature, and vi) variations between instruments (Möller, 2013).

Useful statistics for evaluating NIR spectroscopy calibrations

NIR spectra are complicated and their interpretation is not simple. Chemometrics is the field of extracting information from multivariate chemical or spectral data, using tools of statistics and mathematics. In spectroscopy, the principal application of chemometrics is in the calibration. The variable that calibrations are developed for are referred to as constituents or an analyte. The concentration of the constituents is determined by a standard analytical procedure (Walker & Tolleson, 2010).

Chemometric calibration demands the development of a calibration model, usually by applying one or other mathematical algorithm to the data for which the model is intended to be used (Mark, 2012). Several comprehensive software packages are available, which are dedicated to make use of NIR spectroscopic information and for the development of calibrations. Chemometrics applies statistical methods such as multiple linear regression (MLR), partial least squares (PLS) and principle component analysis (PCA) to the spectral data and correlates them with a physical property or other factor, which is directly determined rather than the analyte concentration itself. PCA and PLS can be considered standard calibration techniques for NIR spectroscopy equations. The main advantage of these techniques is to avoid co-linearity problems permitting to work with a number of variables that is greater than the number of samples. A comparison between these two techniques reveals similar results in terms of prediction performance, with no significant

difference being reported when both employ the optimised number of principal components (PC's). PLS usually produces good models using a lower number of PC's than its counterpart, PCR. All methods assume a linear relationship between the spectral data and the concentration or other property value to be determined. The primary method provides the data of the wet chemistry of the samples required to develop the calibration, although the actual measurement when a sample not used in the calibration set is scanned in the NIR spectroscopy instrument is a prediction based on the statistics of the data, not on the direct quantification of the analyte (Ruiz, 2001; Pasquini, 2003; Williams, 2012).

The program correlates special characteristics of the calibration samples with the respective reference data to derive the model. The model can then be applied to spectra of unknown samples to yield an analytical result (Mark, 2012).

NIR spectroscopy instruments can be calibrated by relating spectra, which are obtained by using a set of known samples, to reference chemical data for the same set of samples. The chemical and physical properties of unknown samples of the same type of material can be used to predict the unknown samples by using the obtained calculation (Graham *et al*., 2013). The efficiency of NIR spectroscopy calibrations is usually evaluated by means of applied statistics. The efficiency of a regression equation for a set of calibration samples can be reported by including the following statistics: standard error of calibration (SEC), the coefficients of correlation (*r*), coefficients of determination (r^2) and regression (b), the intercept (a) and bias, as well as the standard error of prediction (SEP), which is the standard deviation (SD) of the differences between NIR spectroscopy predicted values and reference values. The higher the value of r^2 and the lower the SEP, the more effective is the calibration (Williams, 2001).

Several terms are needed for the interpretation of statistical analysis of the results of NIR spectroscopy calibration equations. Unless all of them are understood and correctly interpreted, the operator may draw the wrong conclusions, which can lead to frustrating and sometimes costly discrepancies. To assess the quality of a calibration model, several standard statistical measures are useful to describe the performance of the developed models and for comparative studies of different models (Malm *et al*., 2012).

These statistics for interpretations of NIR spectroscopy for computing calibrations include:

(i) The mean of the independent variable/spectral (log 1/R) data (X bar); the mean of the dependant variable/ reference data (Y bar) (where X is the value determined by conventional analytical methods and Y is the value predicted by NIR spectroscopy)

(ii) The standard deviation (SD) of both the independent (SD_x) and dependent (SD_y) variables data for the samples used in calibration and validation monitoring

(iii) The coefficient of variability or variation (CV) calculated from the SD and X bar

(iv) The bias, i.e., the mean difference between X and Y data

(v) The coefficient of correlation (*r*) between X and Y data

(vi) The coefficient of determination (r^2)

(vii) The regression coefficient (b) and intercept (a)

(viii) The distribution of differences between X and Y (in NIR spectroscopy it is usually referred to as predicted NIR spectroscopy values and reference values)

(ix) The standard error of a single test (SET) or precision

(x) The standard error of prediction or standard error of performance (SEP)

(xi) The standard error of cross-validation (SECV)

(xii) The root mean square deviation of differences (RMSD)

(xiii) The true test error (TTE)

(xiv) The ratio of the SEP to the SD_y (RPD)

(xv) The ratio of the range to the SD_y (RER) (Williams, 2007).

The development of a calibration model on NIR spectroscopy requires a data set with spectra and corresponding reference values measured analytically on a representative sample set. The spectroscopic data are designated by the letter X and the reference value by the letter Y (Malm *et al.,* 2012). The coefficient of correlation (*r*) shows the degree to which two sets of data (X and Y data, e.g. the NIR spectroscopy predicted values and reference values) correlate with each other. Perfect correlation, with no differences at all between the two data sets, will result in an r-value of 1.000. In practice this is impossible, since a certain amount of error in either X or Y data (or both) is unavoidable. The X and Y

data may either be positively or negatively correlated. The coefficient of determination is given by r^2. It shows the proportion of the variance in X data that can be explained by the variance in the Y data (Williams, 2007).

Table 1 Guidelines for interpretation of *r* and r^2 (Williams, 2001)

***r* value**	**r^2**	**Interpretation**
Up to 0.50	Up to 0.25	Cannot be used in NIRS calibration
0.51 - 0.70	0.26 - 0.49	Poor correlation. Investigation is necessary
0.71 - 0.80	0.50 - 0.64	Can be used for rough screening
0.81 - 0.90	0.66 - 0.81	Can be used for screening and some approximate calibrations
0.91 - 0.95	0.83 - 0.90	Can be used in most applications but with caution
0.96 - 0.98	0.92 - 0.96	Can be used in most applications, including quality assurance
0.99+	0.98+	Excellent - can be used in any application

The coefficient of correlation (*r*) is an indication of the closeness of fit between the NIR spectroscopy and reference values over the range of composition. A high *r* value with a low SEP and bias, together with a slope close to 1.0, indicates that the NIR spectroscopy results are accurate over the anticipated range and likely to remain so, provided that these statistics were based on a sufficient number of observations (Williams, 2001).

The root mean square error of prediction (RMSEP) is a measure for the accuracy of the calibration. The RMSEP is the total error calculated as the square root of the average squared difference between reference values and the predicted values by a regression model when applied to a set of samples which are not included in the derivation of the model. It should be noted that RMSEP includes any bias in the predictions (Malm *et al*., 2012).

The standard error of calibration (SEC) is calculated by the results of prediction of the samples used in the actual development of the calibration and is obtained from the SD of differences between NIRS and reference samples used in the calibration sample set. The spectral signals at adjacent wavelengths are highly correlated with each other and if MLR is used the *r* and SEC statistics will progressively improve as more terms are added. This is called over-fitting of data (multi-collinearity) and can be misleading. If the validation

exercise indicates that *r* is low and the SEP is unacceptably high, the calibration set can be predicted and the individual data viewed. The SEC may indicate the presence of one or more gross outliers, the removal of which may bring about a significant improvement in the actual *r* and SEP values when the validation set is predicted. The SEP should theoretically always be higher than the SEC. Validation using a separate set of samples enables the operator to optimize the number of constants to use in the MLR equation (Williams, 2007).

The SEC may be calculated as follows (Smith & Flinn, 1991):

$$SEC = [\Sigma(Xi - Yi)^2 / (N - p - 1)]^{0.5}$$

where: Xi = the value determined by conventional analytical methods of the calibration set
Yi = the value predicted by NIRS of the calibration set
N = number of samples of the calibration set
p = number of terms in the equation

The standard error of prediction (SEP), also termed standard error of performance, is the standard deviation of differences between NIRS predicted values and the associated reference values, after correction for bias. Unlike the root mean square of the differences (RMSEP), the SEP is independent of bias. The SEP should be calculated from the results obtained by predicting a set of samples that have not been used in development of the calibration. This sample set is usually termed the prediction set, or more often, the validation set. Ideally, the sample set used in validation of a calibration, should consist of samples of the same type that are not related to the calibration sample set. Often, the validation samples are part of a single population from which both the calibration and validation samples sets are compiled (Williams, 2007).

In practice, the SEP may not always be higher than the SEC. For some applications, the precision of the NIRS instrument may be superior to that of the reference method. Sample selection for calibration and validation set may result in one or more of the calibration samples having a higher reference test error than any of those in the validation set, and the SEP may be slightly lower than the SEC.

The SEP is defined as (Smith & Flinn, 1991):

$$SEP = [\Sigma(Xi - Yi)^2 / (N - 1)]^{0.5}$$

where: Xi = the value determined by conventional analytical methods for the validation set
Yi = the value predicted by NIRS for the validation set
bias = difference between overall means
N = number of samples of the validation set

The calibration set may contain one or more outliers, which will account for the SEP being somewhat lower than the SEC. These can be identified by displaying the correlation plot of the calibration sample set. If the SEP is much higher than the SEC (e.g., SEP is about two times higher than SEC), then there has either been a high degree of over-fitting, or there is a major error in at least one of the samples used in validation (Williams, 2007). The standard deviation (SD) is an expression of the variability, or variance in data. It is the square root of the variance (Williams, 2006).

The RPD (ratio of prediction of deviation) can be defined as the ratio of the standard deviation of results of reference analysis and the standard error of performance of NIRS data (SD/SEP). The RPD relates the SEP to the SD and simplifies the interpretation of the SEP (Williams, 2001). The RPD is a simple statistic used to illustrate the efficiency of the calibration in terms of the original standard deviation of the percentages of the constituent in the series of samples as determined by wet chemistry reference methods, when analysed by NIR spectroscopy. It is calculated by dividing the SD of the reference values used in the validation (SD) by the SEP. When the value of the standard error of performance (SEP) approaches that of the standard deviation (SD), the calibration is not measuring/ predicting anything. Therefore, the higher the RPD value, the more efficient or better the calibration. Ideally, an RPD value of 10 or higher indicates a very good calibration, while values lower than 10 may reflect a poor calibration or too narrow a range in the constituent in the calibration samples. An RPD value of 10 indicates that the error of prediction by NIR spectroscopy is only one-tenth of the standard deviation for the reference result (Panford *et al*., 1988).

The RPD is defined as (Williams, 2001):

$$RPD = \frac{SD}{SEP}$$

The efficiency of NIR spectroscopy predictions is determined by the size and consistency of deviations from analyses (SEP). If the SEP value is similar to the SDx, it means that the instrument is not predicting the reference values at all. The SEP should be much lower than the SDx, and ideally the RPD should be 5 or higher. As shown in Table 2, Williams (2001) provided ranges for the RPD values related to the calibration suitability: values greater than 3 are useful for screening, values higher than 5 indicate effective NIR spectroscopy predictions, values above 8 indicate that the calibration can be used for any application, while values below 2.3 indicate a poor calibration performance, with use for the prediction of new samples not advisable. The calibration equations that are developed must be validated against another data set in which reference values of the constituents have been determined. The samples used in the validation set are normally different from those which were used to develop the calibration equation, and are usually a smaller set than the calibration set. The predicted values will normally be different from the reference values (Dryden, 2003). Criteria have been provided by Williams (2001) to interpret the values for bias, SEP and correlation between predicted and reference values. It was recommended that the SEP should not be more than 3% of the mean reference value for the constituent.

Errors in the reference values are a result of using different subsamples to perform the NIR spectroscopy predictions and reference values and from random and systematic errors in the reference methodology (Hruschka, 2001). Analytical methods should be standardised as much as possible to reduce random error. The amount of error in the reference analysis can be expressed as the laboratory standard error (SEL).

The SEL is defined as (Smith & Flinn, 1991):

$$SEL = [\Sigma(X_1 - X_2)^2 / N]^{0.5}$$

where: X_1 and X_2 = the duplicate analyses for the sample

N = number of samples

Table 2 Guidelines for interpretation of RPD (Williams, 2001)

RPD value	Classification	Application
0.0 - 2.3	Very poor	Not recommended
2.4 - 3.0	Poor	Very rough screening
3.1 - 4.9	Fair	Screening
5.0 - 6.4	Good	Quality control
6.5 - 8.0	Very good	Process control
8.1+	Excellent	Any application

The correlation coefficient should not be below $r = 0.60$, as the calibration will not be accurate enough to be used to predict the values on NIR spectroscopy, because only three to four separate segments are statistically different. Once an NIR spectroscopy instrument is calibrated against a reference method, it can be used to determine the percentage of a constituent in different samples of the same type (called unknowns) or to measure some physical quantity of these samples. This NIR spectroscopy measurement should have a measurement error roughly equal to the SEC. However, the NIR spectroscopy measurement error may, in practice, be significantly larger than the SEC. Comparison between NIR spectroscopy measurement and reference methods measurement on a new set of samples provides a basis for calculation of the true measurement error. This comparison is called the validation or verification of the calibration (Hruschka, 2001).

The data obtained from NIR spectra contains background information and noises besides samples information. In order to obtain reliable, accurate and stable calibration models, it is very necessary to pre-process spectral data before modelling (Cen & He, 2007) and it can often improve the calibration accuracy, as NIR spectra are affected by particle size, light scatter and path-length variation. Pre-processing methods include smoothing, standard normal variate (SNV) with, or without detrending (SNV-D), multiplicative scatter correction (MSC) and derivatization. Detrending removes the linear and quadratic curvature of each spectrum; SNV scales each spectrum to have a standard deviation of 1.0 and MSC expands or contracts each spectrum and shifts it up or down to look most like the target spectrum (usually the mean of file of spectra). First or second derivative mathematical treatments are most frequently employed (Deaville & Flinn, 2000).

Another pre-processing method includes the first and second derivative which is used to remove background and increase spectral resolution. The two algorithms to be used during derivatisation include direct differentiation and Savitzky-Golay. The algorithm used most often for differentiation is the Savitzky-Golay, where the data within a moving window are fitted by a polynomial of a given degree to generate a differential of a chosen degree (Cen & He, 2007).

Use of NIR spectroscopy for the prediction of the nutrient composition in oilseeds

Oilseed crops are grown primarily for the oil content in the seeds. The oil content of small grains (e.g. wheat) is only 1 - 2%, compared to that of oilseeds which range from about 20% for soybeans to over 40% for sunflower seeds and canola. The major world sources of edible seed oils are soybeans, sunflowers, canola, cotton and peanuts (Stefansson, 2013).

The characteristic feature of oilseeds is the high content of oil, which is normally about 20% or higher. The residue of the oil pressing process is less important and contributes minimally to the value of an oilseed. Oilseeds are an important economical factor in the world trade of agricultural products. Knowledge of the oil content of the seeds is of key interest to the oil milling business because the monetary assessment in the trade of oilseeds is based on this value. The raw material price depends on its oil content (Matthäus & Brühl, 2001).

The study and evaluation of the oil content of oilseeds are important criteria, especially for the oil milling trade. Standard methods for the determination of the oil content of oilseeds have been the direct solvent extraction method, used since about the 1880's. The method is very time consuming, involving the use of flammable solvents with extraction periods of 4 to 8 hours (Matthäus & Brühl, 2001). Moreover, a sample is destroyed, which is an inconvenience, particularly for plant breeders who often have only a few seeds available for planting and analysis. These serious drawbacks resulted in the development of wide-line nuclear magnetic resonance (NMR) and near infrared reflectance spectroscopy techniques (Robertson & Barton, 1984).

The development of a rapid, accurate and robust instrumental method to evaluate the oil content in oilseeds is of major interest to growers, processors and oilseed breeders. NIR spectroscopy is routinely used for the prediction of oil content in canola crops (Greenwood *et al*., 1999). Pérez-Marín *et al.* (2004) compared different sample modes,

such as milled versus unmilled samples, in order to demonstrate that the analysis of unmilled samples is also possible, thus saving time by not having to mill samples, which is a time consuming process.

The seed quality in oilseed crops is mainly determined by the fatty acid composition of the seed oil. This is commonly analyzed by gas chromatography (GC) of fatty acid methyl esters (Velasco *et al*., 1999). Conventional analytical methods for the determination of fatty acids involve the extraction of fat with diethyl ether or a mixture of chloroform and methanol, followed by conversion of the fatty acid to their methyl esters and analysis by capillary gas chromatography. This procedure is tedious and generates hazardous waste (Pla *et al*., 2007).

An alternative approach is desired that is faster and more cost effective than GC, but also non-destructive and reliable (Velasco *et al*., 1999). NIR spectroscopy has been successfully applied as an alternative technique to gas chromatography for the analysis of fatty acids in a number of oilseed crops, such as whole canola seeds (Pallot *et al*., 1999), sunflower intact seeds, husked seeds, meal and oil (Pérez-Vich *et al*., 1998), *Vernonia galamensis* (Baye & Becker, 2004), safflower (Rudolphi *et al.*, 2012) and Ethiopian mustard (Velasco *et al*., 1996). NIR spectroscopy is an accurate, fast and non-destructive technique which requires little or no sample preparation. Furthermore, NIRS requires no reagents and no waste is produced, as in the case of traditional chemical analysis (Pla *et al*., 2007).

The NIR spectroscopy technique was investigated for the estimation of the relative concentration of the major fatty acids in whole canola seed samples, but results obtained were not accurate enough to enable the routine use of NIR spectroscopy. Although the results obtained in whole canola seed samples were not accurate enough to permit good predictions of fatty acid composition, it revealed that NIR spectroscopy can be used as a rapid and non-destructive screening technique for pre-selecting samples with extreme values to be confirmed with further GC analysis. This screening strategy is especially useful when several thousands of samples have to be analyzed (Daun *et al.,* 1994).

Sato *et al*. (1995) demonstrated that NIR spectroscopy can be applied successfully for the prediction of fatty acid concentration in husked sunflower seeds. Perez-Vich *et al*. (1998) found that predictions of intact sunflower seeds by NIR spectroscopy is a rapid and cost-effective tool for screening of oil content, palmitic acid, palmitoleic, stearic acid, oleic acid and linoleic acid in intact seeds, husked seeds, meal and sunflower oil. Results

obtained by Velasco *et al*. (1999) demonstrated that NIR spectroscopy predictions of intact, single achenes of sunflower for oleic acid and linoleic acid concentrations are accurate for screening sunflower achenes for oleic acid and linoleic acid. Furthermore, the above authors demonstrated that, although with lower accuracy, the concentrations of these fatty acids in the seed oil can also be estimated with a high degree of reliability by analysing bulk samples of intact sunflower achenes.

Kaffka *et al*. (1982) investigated the possibility to determine the oil, protein, moisture and fibre content in sunflower seeds by NIR spectroscopy. They concluded that the NIR spectroscopy technique has the potential for use in rapid evaluation of sunflower seed quality, with correlation coefficients of 0.998 for fat, 0.993 for protein, 0.998 for moisture and 0.991 for fibre.

Non-destructive NIR spectroscopy analysis will therefore enable rapid and reliable selection of materials with different nutritive value and composition of fatty acids in oilseeds such as sunflower seeds and canola seeds.

Use of NIR spectroscopy for the prediction of forage quality

The prediction of the nutritive value of pastures provides an opportunity for formulating a diet with a balance of nutrients which are able to meet the requirements of livestock feeds. The formulation of a balanced diet must be based on the nutritional requirements of the animal for the intended level of production, as well as on the basis of the composition of the potential feed composition. This information can be provided by agricultural professionals, but complex decision making is assisted by a ration balancing model (Corson *et al*., 1999).

The nutrient composition of forage crops is related mainly to climatic conditions and stage of plant maturity and the determination of the nutritive value is important in many pasture experiments, animal feeding trials and extension services. Worldwide, the nutritive value of forages is often estimated by chemical or physical methods, and is expressed as the concentration of chemical constituents in plant tissue (Givens & Deaville, 1999).

Improvements in crop and forage quality often entail screening of large numbers of samples, and this may limit the size of a plant breeding program. NIR spectroscopy appears to be an attractive alternative to routine chemical analysis, resulting in substantial improvements in the efficiency of the breeding process for some aspects of quality such as protein and digestibility (Garcia & Cozzolino, 2006).

Numerous studies have examined NIR spectroscopy for measurement of the composition and functional aspects of forages. NIR spectroscopy can be utilised to screen for a wide range of chemical components, e.g., dry matter (DM), ash, crude protein, acid detergent fibre (ADF), neutral detergent fibre (NDF). In forage research the most frequent applications are to analyse for the fibre, NDF and protein content in grasses, legumes and herbs. NIR spectroscopy applications are restricted by the capacity for accurate calibration and sample characteristics which are able to provide interpretable spectra (Corson *et al*., 1999).

Alfalfa (*Medicago sativa* L.) is one of the most important forages that are cultivated around the world, and the making of hay is the most important method of preservation. The feeding strategy based on alfalfa forage needs a fast and reliable determination of the chemical composition, which is dependent on the cultivar and many environmental factors. Traditionally, wet chemical analysis has been used to determine the nutritive value of forages, but these methods are time-consuming, expensive and sometimes hazardous chemicals are used (Brogna *et al*., 2009). NIR spectroscopy has become a widely recognized tool to determine the nutritive value of a wide range of forages (Shenk & Westerhaus, 1995).

Reliable predictions of forage energy content are needed to formulate rations properly for lactating dairy cows and other ruminants (Lundberg *et al*., 2004). Since Norris *et al.* (1976) reported for the first time that NIR spectroscopy can be used to predict the nutritive value of forage species, NIR spectrosocpy hardware and calibration techniques have been improved and NIR spectroscopy is routinely used to measure feed quality in many parts of the world (Smith & Flinn, 1991).

Garcia & Cozzolino (2006) reported that relatively high correlation coefficients (r^2) and low SECV for DM (0.95; 0.70), ash (0.90; 0.99), crude protein (0.98; 0.98), ADF (0.95; 2.0), NDF (0.86; 5.4) and IVOMD (0.90; 3.0) could be obtained in different forage plant species, but better calibration equations can be obtained if models are developed for single species. They reported that NIR spectroscopy calibration models indicated good correlations with all the chemical constituents which were analyzed ($r^2 > 0.90$), although the lowest r^2 were obtained for NDF ($r^2 = 0.86$).

Chemical composition of animal feed samples

Compound feed mixes are composed of a wide variety of feed ingredients incorporated into a basic feed ration. Daily price fluctuations in commodities are the main reason for variances in feed ingredients used in diets. The feed ingredients most frequently used in the feed industry include cereals, such as wheat, barley, maize and oats. Protein sources include soya bean and rapeseed meals, meat meal and fishmeal. Roughage is provided by adding hay and other forage such as alfalfa (lucerne). Vitamin and mineral premixes and antibiotics are also included as additives. Due to variances in average particle size, particle size distribution and bulk density, the slopes of instrument response to composition show significant differences between commodities (Williams & Starkey, 1980).

Traditional analytical methods are still being used for the analysis of animal feeds, although a few studies have been done with NIR spectroscopy calibrations on the feed composition of different complete animal feeds. Satisfactory results were obtained in rabbit feed by Xiccato *et al*. (1999), commercial pig feed mixes (Chen *et al*., 1987), complete poultry feeds by Valdes & Leeson (1992) and compound feeds for swine and ruminants (Aufrère *et al*., 1996). De Boever *et al*. (1995) obtained accurate calibrations to predict the moisture, crude protein, crude fat, crude fibre and energy content in compound feeds for cattle, but not accurate calibrations for the prediction of the ash content. Inorganic substances, which were added in the form of minerals and trace elements, do not absorb from the near infrared wavelength region. Pérez-Marín *et al.* (2004) developed NIR spectroscopy equations to predict the chemical and ingredient composition of compound feeding stuffs for different types of animals. Calibrations for the prediction of chemical composition in total mixed rations showed similar accuracy for the different modes of analysis assayed.

Calibrations to predict the nutritive value of raw materials are more accurate than calibrations for compound feeds, as they are constituted of a large range of raw materials which may exhibit different spectral characteristics for apparently the same chemical component (Givens & Deaville, 1999).

Studies done by de Boever *et al*. (1995) prove that the chemical composition and the energy value of complete feeds for cattle vary considerably because of the wide choice of raw materials and by-products and the variable needs for supplementing the basic diet. The farmer needs to know the quality of the concentrates he purchased, not only for

economic reasons, but also from a feed-technical and ecological viewpoint. To achieve the production potential of animals and to avoid unnecessary mineral losses to the environment, it is essential to adjust the nutrient supply to the individual requirements of the animals.

In a study done by González-Martín *et al.* (2006a), it was found that NIR spectroscopy with a remote reflectance fibre-optic probe can be used as a quality control method in animal feeds and fodder for the determination of crude protein, ether extract, and crude fibre. The samples had different physical characteristics in the form of meal, tablets and granules, and were used in different stages of animal development for cattle, pigs, sheep, poultry and rabbits.

Mentink *et al.* (2006) evaluated the use of NIR spectroscopy to predict the chemical and biological nutrients in total mixed rations. The study was conducted to evaluate technical aspects of currently available commercial programmes that evaluate nutrients in total mixed rations by making use of NIR spectroscopy. Good results were obtained to predict the basic nutrients, such as crude protein, neutral detergent fibre (NDF), starch, non-fibre carbohydrates (NFC) and ether extract in total mixed rations. The development of NIR spectroscopy calibrations to predict these nutrients in total mixed rations can be used to monitor mixing of feeds and basic aspects of feed formulations. The authors could not obtain good calibrations to predict biological nutrients, such as *in vitro* NDF digestibility and the *in situ* protein fractions by NIR spectroscopy, which can be explained by reference method error in relationship to the range of nutrient values available. Better NIR spectroscopy equations can be obtained if more accurate laboratory procedures or multiple laboratory replications can be used.

Amino acids are important components in the composition of animal feeds and knowledge of their levels and control of these allows the nutritional value of feeds to be evaluated and enables determination of the correct amounts of them to be added to feeds. However, the chemical determination of amino acids by high pressure liquid chromatography (HPLC) is a long and time consuming analysis. It involves the hydrolysis of protein in the sample and analysis by means of an HPLC, which is an accurate method, but involves a complex, expensive instrument and a tedious process. Results indicated that amino acids, such as alanine, aspartic acid, glutamic acid, glycine, phenylalanine, valine, proline and tyrosine can successfully be determined by NIR spectroscopy with remote reflectance fibre-optic probe. The results obtained by NIR spectroscopy was

successfully applied on feed samples with different textures, such as blocks, tablets, granules and meal, which are used in different growth stages of the animal feeding programmes, such as lactation, growth and maintenance and are comparable with results obtained with the chemical ion-exchange HPLC method (González-Martín *et al*., 2006b).

Conclusion

NIR spectroscopy has had rapid usage growth since its first application in the 1960s in the grain industry. Since then, NIR spectroscopy applications have successfully been reported in the material science, food, environment, medicine, pharmaceutics, agriculture and archaeology (Agelet & Hurburgh, Jr., 2010). NIR spectroscopy is widely used for rapid and economical measurement of feedstuff ingredients, forage foods and concentrates for both monogastric and ruminant animals. A wide range of nutritionally important constituents (e.g. proteins, fibres, starches and sugars) and related functional properties (e.g. digestibility and voluntary intake by the animal) of feedstuffs and forages can be measured from their absorption characteristics (Dryden, 2003).

NIR spectroscopy technologies offer fast solutions for organic compound discrimination and quantification. With the instrumental market in constant growth and development, cheaper and yet more accurate instruments will probably offer opportunities to explore new applications and field of work. However, choosing a suitable instrument for an application involving the use of NIR spectroscopy is not even half of the requirement for its success. Sample selection, chemometric methods and validation are key factors that should not be overlooked (Agelet & Hurburgh, Jr., 2010).

The methods of NIR spectroscopy analysis of protein, moisture and hardness in grains and the determination of protein, ADF and moisture in forages, have been accepted by the International Standards Committees. The reasons for NIR spectroscopy being adopted in many laboratories as the preferred analytical method over wet chemistry analyses include that minimal sample preparation is required, that it is fast to analyse a sample and is cost effective to analyse a single sample or large batches of samples. Several components can be determined simultaneously and the samples are not destroyed during analyses. The operation of the instrument does not require a skilled operator and no hazardous chemical reagents are used (Batten, 1998).

In all the applications that have been discussed, NIR spectroscopy predictions can only be as good as the calibration data which is obtained from the reference samples, but

once calibration equations are developed, NIR spectroscopy offers fast and economical analysis, which is capable to screen a large number of samples (Corson *et al*., 1999).

Aim of the study

The aim of this study is to evaluate the usefulness of the NIR spectroscopy technique for the evaluation of feed quality with regard to dry matter (DM), ash, crude protein, crude fibre, crude fat, acid detergent fibre (ADF), neutral detergent fibre (NDF), calcium (Ca), phosphorus (P), *in vitro* organic matter digestibility (IVOMD), gross energy (GE), amino acids such as lysine, methionine, threonine and arginine as well as fatty acids such as palmitic acid, stearic acid, oleic acid and linoleic acid in feed samples as well as feed ingredients such as alfalfa, canola, lupins and sunflower seeds.

The objectives of this study were to (i) predict the dry matter, ash, crude protein, crude fibre, oil content, palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2) of sunflower seeds using NIR spectroscopy, (ii) determine the use of NIR spectroscopy to predict the chemical composition of milled canola seed compared to whole canola seed, (iii) predict the chemical composition of milled and whole lupin seeds with NIR spectroscopy, (iv) evaluate the use of NIR spectroscopy to predict the chemical composition and amino acid content of alfalfa hay, and (v) determine the use of near infrared reflectance spectroscopy to predict the chemical composition and amino acids of ostrich total mixed ration samples.

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CHAPTER 2

PREDICTING THE CHEMICAL COMPOSITION AND FATTY ACID CONTENT OF SUNFLOWER SEED MEAL USING NEAR INFRARED REFLECTANCE (NIR) SPECTROSCOPY

Abstract

The estimation of the nutritional profile of seeds by near-infrared reflectance (NIR) spectroscopy allows for non-destructive predictions which are highly desirable in plant breeding. The major advantage of NIR spectroscopy is that it is a non-destructive analytical method; therefore, it requires no chemical reagents, and once the calibrations are developed, it takes just minutes or a few seconds to have a result of one or more constituents, which by traditional chemical analysis may take hours or days. NIR spectroscopy was explored as a technique to predict the dry matter, ash, crude protein, crude fibre, oil content, palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2) (an essential fatty acid) content of sunflower seed meal. Sunflower seeds with a large range in variation in their fatty acid composition were used to develop the calibration equations. A total of 160 samples were used for the dry matter (DM), ash, crude protein, crude fibre and oil content calibrations and a total of 100 samples were used for the fatty acid calibrations from a sunflower breeding program. The sunflower seed meal samples were analysed with reference analyses (AOAC and gas chromatography) and scanned on a Bran & Leubbe InfrAlyzer 500. The four major individual fatty acids (C16:0, C18:0, C18:1 and C18:2) were analysed as fatty acid methyl esters (FAME) on a Thermo Focus gas chromatograph. Calibration equations were developed using modified partial least square regression (MPLS) with external cross validation. NIR spectroscopy calibration equations were developed and tested through external cross validation. The coefficient of determination in calibration (r^2_{cal}) and the standard error of calibration (SEC) for sunflower seed meal for DM were 0.71 and 0.44%, ash 0.56 and 0.40%, crude protein 0.87 and 0.93%, crude fibre 0.83 and 1.98% and for oil content 0.85 and 1.29%, respectively. The corresponding values for palmitic acid were 0.40 and 2.95 mg/g, 0.45 and 3.78 mg/g for stearic acid, 0.78 and 16.58mg/g for oleic acid and 0.87 and 12.43 mg/g for linoleic acid, respectively. The coefficient of determination of the external cross validation was the highest for crude protein and the lowest for palmitic acid. The results

indicate that NIR spectroscopy calibrations are applicable in sunflower breeding programmes for a fast screening. Screening of sunflower seeds by NIR spectroscopy represents a rapid, simple and cost effective alternative that is a great utility for users who need to analyse a large number of samples.

Keywords: sunflower seed meal, fatty acid composition, nutritive value

Introduction

Sunflower (*Helianthus annuus* L.) is one of the fastest developing oilseed crops in the world and is the fourth largest source of oilseeds after soybean, palm and canola (Stefansson, 2013). Sunflower is also appreciated as a high quality commodity in the world oil market (Fernández-Martínez *et al*., 2007). Furthermore, sunflower seeds are one of the important dual purpose crops in the world as it provides both protein and oil. As the most important source of vegetable oil in South Africa, sunflower is predominantly cultivated in the summer rainfall areas and annual production ranges between 500 000 to 700 000 tons (Dredge, 2014). The average sunflower seed yield ranges from 1.2 to 1.8 ton/hectare under dry land conditions, resulting in the production of sunflower to be the third largest grain crop after maize and wheat in South Africa. Regardless, South Africa is not a significant role player in the international market, contributing only 3% to the sunflower seed produced in the world (Anonymous, 2014). Sunflower is primarily grown in South Africa as a source of vegetable oil and the area under cultivation of sunflower is continuously increasing (Muya, 2012). Sunflower and sunflower oilcake meal remains an important source of protein to supply the South African demand. Until two years ago, when soybean production surpassed sunflower production for the first time, sunflower and sunflower oilcake meal were the main local source of protein for animal consumption (PRF, 2013).

The three main uses of sunflower are: (1) for the production of sunflower oil, which is used for human consumption, (2) as animal feed, and (3) for numerous industrial uses. When used as a food source for human consumption, it is widely applied in the form of visible fat (margarine, salad dressing oil, and cooking oil) and invisible fat (milk, meat, cheese, pastry, snacks, bread, nuts) (Moschner & Biskupek-Korell, 2006; Fernández-Martínez *et al*., 2007). As animal feed, the whole seeds or partly dehulled sunflower meal can be used for ruminant animals, pigs and poultry feeds because of its high fat and

protein content and the silage can be used for animal feeds. The seeds and oil cake meal is favoured as animal feeds as it has been found to have a high protein (32 - 35%) and oil content (37 - 45%). The fatty acid profiles of the oil are important for both human, as well as animal nutrition and health. Sunflower is a widely cultivated oilseed, although utilisation in poultry diets is limited because it has a high fibre content and low lysine content, compared to other oilseeds, especially soybeans (San Juan & Villamide, 2000). The industrial use of sunflower includes the use in certain paints, varnishes and plastics due to the good semi-drying properties without colour modification associated with oils high in linoleic acid (Anonymous, 2014). Other industrial uses include the production of motor fuels (biodiesel) and lubricants, as well as many applications in the oleochemical industry (detergents, soaps, surfactants, emulsifiers, cosmetics, etc.), agrichemicals or pesticides, surfactants, adhesives, fabric softeners, lubricants and coatings (Fernández-Martínez *et al*., 2007).

Due to the high levels of unsaturated fatty acids that relates to good nutritional characteristics for healthy living of any livestock, the consumption of vegetable oils is very important. Consequently, one of the major objectives for breeding sunflower seeds is to incorporate these healthy oils into food sources and thereby improving the quality thereof. This is of particular importance when refining the quality of animal feed earmarked for breeding programmes. In such cases, the analysis of the fatty acid composition of sunflower seed oil is required (Sato *et al*., 1995).

Several types of sunflower oils are produced, such as high linoleic, high oleic and mid linoleic. Mid linoleic sunflower oil typically has at least 69% linoleic acid and high oleic sunflower oil has at least 82% oleic acid. For the profitable use of high-oleic sunflower oil in food and non-food areas, it is very important to have rapid and accurate analytical methods to determine quality parameters. Nutritional properties of sunflower oils are determined by their fatty acid profile, the distribution pattern of the fatty acids within the triacylglycerol molecule, and the total content and composition of natural antioxidants, especially tocopherols and the free fatty acids (FFA) of the harvested and trades high-oleic sunflower seeds (Fernández-Martínez *et al*., 2007; Moschner & Biskupek-Korell, 2006).

Oleic acid (a mono-unsaturated fatty acid) content of oilseeds has important implications for product performance and consumer health (Tillman *et al*., 2006). High linoleic acid oils have alternative nutritional advantages such as the production of

conjugated linoleic acid (CLA), which is associated with a wide range of positive health benefits (Belury, 1995).

The development of sunflower cultivars with increased levels of saturated fatty acids could increase the usefulness of the oil for specific human consumption purposes (Osorio *et al*., 1995).

Conventionally, the fatty acid composition in samples is determined using gas chromatography (GC). This procedure involves oil extraction, sample preparation for analysis and injecting the sample onto a GC. However, this method is destructive, time consuming, expensive and requires the use of toxic and flammable reagents and gases (Moschner & Biskupek-Korell, 2006).

An alternative approach that is rapid and more cost-effective than GC, as well as non-destructive and reliable, is therefore desired (Velasco *et al*., 1996). Near infrared reflectance (NIR) spectroscopy offers important advantages over traditional chemical analysis in that it is fast, non-destructive and does not require the use of any chemical reagents. A further advantage of NIR spectroscopy is the simultaneous and simple determination of different parameters (Pérez-Vich *et al*., 1998). A basic requirement for the development of NIR spectroscopy calibrations is the availability of a sufficiently dimensioned calibration set. The samples should be from different origins, varieties and harvest years, and should cover a broad range of the nutrients one wishes to develop calibrations for. Once the instrument is calibrated for the specific sample type, it is easy to operate and requires minimal sample preparation (Moschner & Biskupek-Korell, 2006).

Sato *et al*. (1995) demonstrated that NIR spectroscopy could be used to analyse the linoleic acid concentration in bulk, as well as single dehusked sunflower seed samples. Additionally, Pérez-Vich *et al*. (1998) predicted linoleic-, palmitic-, palmitoleic-, stearic- and oleic acid concentrations accurately using NIR spectroscopy in bulk samples of dehusked sunflower seeds.

NIR spectroscopy allows rapid and economical analysis with minimal sample preparation and without the generation of wastes. Although conventional laboratories are still needed to develop and adapt general calibrations for local conditions and maintain NIR spectroscopy calibration equations, the number of samples requiring conventional analytical procedures can be drastically reduced and there is often opportunity to centralise laboratories. Some NIR spectroscopy instruments are designed to be rugged, reliable and portable, which allows the use of the technology away from central

laboratories and in the field, and with minimal training and technical support (Dixon & Coates, 2010).

The aim of this study was to establish whether NIR spectroscopy and appropriate chemometrics could be used for the prediction of various chemical constituents (dry matter, ash, crude protein, crude fibre, oil content, palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2) in sunflower seed meal. The determination of fatty acid concentration in sunflower seeds with NIR spectroscopy would save considerable time, labour and expense, and would allow the evaluation of a large amount of samples, which is more laborious and expensive when using standard chemical procedures.

Materials and methods

Sampling and preparation

The sample set of sunflower seeds (n = 160), kindly supplied by Agricol, consisted of different varieties chosen from a wide range of breeding material of the 2007/2008 harvest season. The samples originated from one locality, i.e. Potchefstroom, North West Province, South Africa. The breeding material differed, among others, with respect to the fatty acid content and included linoleic, mid oleic and high oleic acid. According to Fernández-Martínez *et al*. (2007), sunflower seeds are classified according to the fatty acid concentrations as high oleic (>85%) and high linoleic (>75%). The seeds were stored at 4 - 6 °C in closed containers before milling.

NIR spectroscopy calibration equations for concentrations of chemical composition and fatty acids were developed from a total of 160 sunflower seed meal samples. The chemical analysis of DM, ash, crude protein, crude fibre and oil content was conducted on the entire set of samples and analysis for fatty acids was conducted on 100 randomly selected samples.

Approximately 20 g of sunflower seeds were milled per sample, using a Foss Tecator Knifetec 1095 sample mill (Foss Analytical, Hilleröd, Denmark). The Knifetec sample mill was equipped with a cooling feature attached to the grinding chamber, which enabled the milling of samples containing high levels of fats, such as sunflower seeds, thereby preventing overheating during milling. High fat samples have a tendency to stick to the wall of the chamber as the fat softens during grinding, which prevents adequate homogenisation. The milling protocol was further standardised by keeping the milling

period of 20 seconds constant for all the samples. Sample preparation is an important step in all analytical procedures and it is crucial to grind properly to obtain the highest quality of the analytical results (Foss Analytical, 2005). The important factor in grinding sunflower seeds for NIR spectroscopy analysis is to obtain a finely ground, pourable and homogenous grist in which the pericarp is also micro-milled. However, milling the sample for too long causes the destruction of the oil bodies and increases the sample temperature. Both factors lead to the formation of conglomerates in the meal, which cannot be eliminated by subsequent mixing and have to be avoided. Therefore, it is very important to apply a standardised grinding protocol (Moschner & Biskupek-Korell, 2006).

Analysis of reference samples

The chemical properties of sunflower seeds were determined with the official methods as described by the Association of Official Analytical Chemists (AOAC, 2012). Moisture content was determined by placing (2 ± 0.01 g) milled sunflower seed in a forced-air oven at 100 °C for 24 hours (AOAC, 2012) (Method no: 934.01). The ash content was determined by combustion in a Labcon Muffle furnace RM7 at 500 °C overnight (18 hours), according to AOAC (2012) (Method no: 942.05). Milled sunflower seed samples (2 ± 0.01 g) were placed in a combustion furnace, after which the samples were cooled in dessicators. Nitrogen was determined by the combustion method (AOAC, 2012) (Method no: 990.03). A LECO TruMac N Nitrogen Determinator, Version 1.3X (Leco Corporation, St. Joseph, Michigan, USA) was used and the results were expressed as nitrogen multiplied by the protein factor of 6.25. The oil content was determined by using the official method (AOAC, 2012) (Method no: 2003.06) where a Soxtec system HT 1043 (Tecator, Höganas, Sweden) with diethyl ether as extraction fluid was used. Crude fibre content was determined by using the method of Goering & van Soest (1970), making use of FIWE Raw Fiber Extractor, Velp Scientifica (Velp Scientifica, Milano, Italy). These measurements were done in duplicate and the averaged results were used.

The fatty acids methyl esters (FAME) were determined using a modified method of Folch *et al.* (1957) where chloroform/methanol (CM 2:1; v/v) (30 ml) were used to extract the oil from the milled sunflower seed samples (2 ± 0.01 g). All extraction solvents contained 0.01% butylated hydroxytoluene (BHT) as an antioxidant. To homogenise the sample within the extraction solvent, a polytron mixer (WiggenHauser, D-500 Homogenizer) was used.

Heptadecanoic acid (C17:0) (Sigma-Aldrich Inc., St. Louis, USA) was used as an internal standard to quantify the individual fatty acids. A sub-sample of the extracted lipids was transmethylated and thin-layer chromatography (TLC) was performed. The fatty acid band was observed under long wave ultraviolet light and transmethylating reagent was added to the sample. After being dried under nitrogen, 20 uL carbon disulfide (CS_2) was added and 1 µL analysed by Thermo Focus GC (Thermo Electron S.p.A, Milan, Italy) equipped with a flame ionized detector, using a BPX70 capillary column (60 m x 0.25 mm internal diameter, 0.25 µm film, SGE (SGE International PTY Ltd, Victoria, Australia)). The individual fatty acids were identified by comparing the retention times to those of a standard FAME mixture (Supelco^{TM} 37 Component FAME mix, 10 mg/ml in dichloromethane (CH_2Cl_2), Supelco, Bellefonte, USA).

Collection of spectra and calibration development

The absorbance of reflectance spectra was measured in the NIR region (1100 - 2500 nm) of the electromagnetic spectrum at 2 nm intervals, acquiring 701 data points for each sample. The samples were individually presented in closed cups (approximately 6 g) and the scans were acquired using an InfrAlyzer 500 Near Infrared Reflectance Spectrometer (IA-500). Bran & Leubbe SESAME Version 2.00 software (Bran & Luebbe GmbH, Norderstedt, Germany) was used to perform the spectroscopic measurements (Bran & Luebbe, 1994).

A total of 160 sunflower seed samples were used to develop calibrations for DM, ash, crude protein, crude fibre and oil content. From this sample set, 100 sunflower seed samples were used for the development of palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2) calibrations. The samples were divided into two sets for the different constituents. The larger set was used as the calibration set with n = 134 for DM, ash, crude protein, crude fibre, and oil content and n = 80 for palmitic acid, stearic acid, oleic acid and linoleic acid. The external validation set was the smaller set, with n = 26 for DM, ash, crude protein, crude fibre, and oil content and n = 20 for palmitic acid, stearic acid oleic and linoleic acid which was used to test the accuracy of the calibrations.

Calibration equations were developed by means of partial least square regression (PLSR) on 2nd derivative spectra (segment = 1; gap = 0). As suggested by the SESAME version 2.00 software, the outliers were removed and every fifth sample was selected to

use as the validation set. The second derivative function appeared to be better suited for the estimation of all constituents.

Figure 1 presents the NIR spectra that were collected for 160 sunflower seed samples.

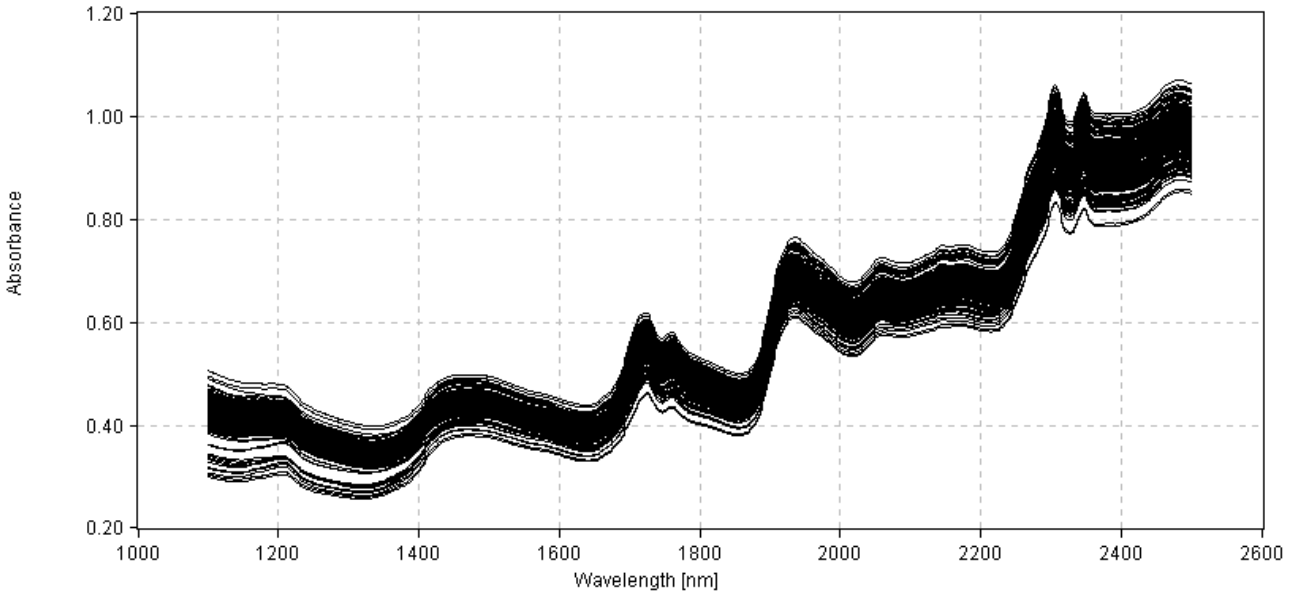


Figure 1 Original near infrared reflectance spectra obtained for sunflower seed meal samples

Results and discussion

NIR spectroscopy calibration equations were developed using partial least square regression (PLSR) with cross validation for dry matter, ash, crude protein, crude fibre, oil content, palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2). Each individual fatty acid was expressed as the milligram per gram (mg/g) sample. The minimum, maximum, mean values and standard deviations (SD) for each constituent for the calibration and validation sets of the calibrations developed for sunflower seed meal are reported in Table 1 and 2, respectively.

The chemical composition of sunflower seed meal samples used in the calibration equations as reported in Table 1 were 93.77% for DM, 3.36% for ash, 18.98% for crude protein, 37.56% for crude fat and 29.89% for crude fibre, which corresponds with values obtained by Srilatha & Krishnakumari (2003) for DM (94.50%), ash (3.49%), crude protein (18.72%), crude fat (37.47%) and crude fibre (28.36%). The mean values for the individual

fatty acids were 15.36 mg/g for palmitic acid, 15.80 mg/g for stearic acid, 75.59 mg/g for oleic acid and 47.93 mg/g for linoleic acid.

Table 1 Summary of nutrient composition (%) and fatty acid content (mg/g) of sunflower seed meal used in the calibration set, showing the number of samples (n), minimum, maximum, mean and standard deviation (SD)

Chemical component/ Fatty acid	n	Min	Maximum	Mean	SD
DM%	134	91.14	97.04	93.77	0.81
Ash%	134	1.56	4.52	3.36	0.61
Crude protein%	134	12.08	26.03	18.98	2.56
Crude fibre%	134	19.87	48.63	29.89	4.83
Oil content%	134	29.76	48.18	37.56	3.38
C16:0 mg/g	80	7.79	24.99	15.36	3.81
C18:0 mg/g	80	6.74	30.73	15.80	5.12
C18:1 mg/g	80	14.97	172.96	75.59	35.17
C18:2 mg/g	80	0.35	117.30	47.93	34.56

The chemical composition of sunflower seed meal samples used in the validation set as reported in Table 2, were 93.84% for DM, 3.35% for ash, 18.78% for crude protein, 37.34% for crude fat and 30.43% for crude fibre. The mean values of the samples used in the validation set for the individual fatty acids were 15.46 mg/g for palmitic acid, 16.24 mg/g for stearic acid, 76.20 mg/g for oleic acid and 48.65 mg/g for linoleic acid. Pérez-Vich *et al.* (1998) investigated a set of 387 sunflower intact seed samples for fatty acid profile. The results revealed the range for C16:0 (palmitic acid) as 3.00 - 35.50%, C18:0 (stearic acid) 1.40 - 30.30%, C18:1 (oleic acid) 7.70 - 90.70% and C18:2 (linoleic acid) 1.80 - 74.50% as a percentage of the total fatty acids. The results revealed that the fatty acid composition of different sunflower oil types (e.g. oils with high palmitic acid, high stearic or high oleic acid contents) can be accurately determined by NIR spectroscopy.

Table 2 Summary of nutrient composition (%) and fatty acid content (mg/g) of sunflower seed meal used in the validation set, showing the number of samples (n), minimum, maximum, mean and standard deviation (SD)

Chemical component/ Fatty acid	n	Min	Maximum	Mean	SD
DM%	26	92.61	96.33	93.84	0.86
Ash%	26	1.87	4.51	3.35	0.58
Crude protein%	26	13.31	25.45	18.78	2.68
Crude fibre%	26	19.87	40.94	30.43	4.79
Oil content%	26	26.31	44.35	37.34	3.37
C16:0 mg/g	20	7.79	24.99	15.46	4.18
C18:0 mg/g	20	6.74	25.91	16.24	4.98
C18:1 mg/g	20	15.81	165.49	76.20	42.24
C18:2 mg/g	20	4.24	139.06	48.65	34.66

Fassio & Cozzolino (2004) have found that NIR spectroscopy has proven that it is capable to produce repeatable results for pre-screening of quality characteristics on intact sunflower seeds for breeding purposes. Although the coefficients of determination in prediction obtained for oil and moisture ($r^2_{cal} < 0.70$) were not high, the advantage of evaluating individual phenotypes in sunflower breeding for quality characteristics outweigh the losses of accuracy when many samples need to be selected.

Results obtained by Velasco *et al*. (2004) indicate that high correlations for stearic acid ($r^2 = 0.83$), oleic acid ($r^2 = 0.92$) and linoleic acid ($r^2 = 0.93$) were found between predicted NIR spectroscopy values and GC analysis, when calibration equations were developed for husked achenes. Velasco *et al*. (1999) reported r^2 in cross validation of 0.91 for oleic acid and 0.92 for linoleic acid and SECV to SD ratios of 0.30 for oleic acid and 0.29 for linoleic acid, which corresponds with results obtained by Velasco *et al*. (2004). These results revealed that the analysis of husked achenes instead of unhusked achenes did not present clear advantages for these fatty acids. NIR spectroscopy can reduce the amount of analysis done by GC, as it is more expensive and time-consuming than NIR spectroscopy. NIR spectroscopy has the additional advantage that the oil content and fatty acid composition can be analysed simultaneously (Pérez-Vich *et al*., 1998).

Hurburgh (2007) states that there are complicating issues for calibration of fatty acids in oil still within its solid substrate. NIR spectroscopy counts molecules of a certain type: the number of certain fatty acid molecules as a percent of oil is affected by the total oil percentage as well as concentration within oil. Spectral influences from many compounds are combined in every scan. The lesser effects (individual fatty acids and individual amino acids) are difficult to separate from the major influences of moisture, total protein, total oil and sugars. Plant breeding efforts created a highly non-normal distribution; samples were either normal, or low, with few in between.

The standard error of calibration (SEC) is calculated by comparing the laboratory values of one set of samples to the instrument values of that same set when creating the calibration equation. The standard error of prediction (SEP) is obtained by comparing the laboratory values of a second set of samples to the instrument percent reading of that second set and verifying the existing calibration (Kaffka *et al*., 1982).

The calibration and external cross validation results were obtained in the calibration equations developed from sunflower seed meal samples scanned for DM, ash, crude protein, crude fibre, oil content, palmitic acid, stearic acid, oleic acid and linoleic acid. The coefficient of determination in calibration (r^2_{cal}) and the standard error of calibration (SEC) for DM, ash, crude protein, crude fibre, oil content, palmitic acid, stearic acid, oleic acid and linoleic acid were determined. The relationship between coefficient of correlation in calibration (r^2_{cal}), SEC, coefficient of correlation in validation (r^2_{val}), SEP and RPD are presented in Table 3, together with the means and standard deviations (SD) of the laboratory and predicted values.

The coefficient of determination in calibration (r^2_{cal}) values was 0.71 for DM, 0.56 for ash, 0.87 for crude protein, 0.83 for crude fibre and 0.85 for oil content. SEC values were 0.44% for DM, 0.40% for ash, 0.93% for crude protein, 1.98% for crude fibre and 1.29% for oil content. The corresponding values for the individual fatty acids were 0.40 and 2.95% for palmitic acid, 0.45 and 3.78% for stearic acid, 0.78 and 16.58% for oleic acid and 0.87 and 12.43% for linoleic acid. In the validation tests the r^2 values ranged from 0.53 for crude fibre to 0.83 for crude protein and SEP values ranged from 0.38% for ash to 3.39% for crude fibre. The corresponding validation statistics for the individual fatty acids was 0.48 and 3.10 mg/g for palmitic acid, 0.51 and 3.49 mg/g for stearic acid, 0.72 and 23.50 mg/g for oleic acid and 0.75 and 17.46 mg/g for linoleic acid. The values for DM, ash, crude

protein, crude fibre, and oil content showed relatively low SEC and SEP values and higher SEC and SEP values was recorded for the individual fatty acids.

Table 3 Calibration and external validation statistics in the development of calibration equations for nutrient composition and fatty acid content in sunflower seed meal samples

Chemical component/ Fatty acid	Calibration set		Validation set			Actual lab values - validation set		NIRS predicted values - calibration set	
	r^2_{cal}	SEC	r^2_{val}	SEP	RPD	Mean	SD	Mean	SD
DM %	0.71	0.44	0.67	0.50	1.72	93.84	0.86	93.77	0.68
Ash %	0.56	0.40	0.59	0.38	1.53	3.35	0.58	3.36	0.46
Crude protein %	0.87	0.93	0.83	1.10	2.44	18.98	2.68	18.98	2.38
Crude fibre %	0.83	1.98	0.53	3.39	1.41	30.43	4.79	29.89	4.41
Oil content %	0.85	1.29	0.64	2.27	1.48	37.34	3.37	37.56	3.13
C16:0 mg/g	0.40	2.95	0.48	3.10	1.35	15.46	4.18	15.36	2.40
C18:0 mg/g	0.45	3.78	0.51	3.49	1.43	16.24	4.98	15.83	3.45
C18:1 mg/g	0.78	16.58	0.72	23.50	1.80	76.20	42.24	75.59	30.98
C18:2 mg/g	0.87	12.43	0.75	17.46	1.99	48.65	34.66	48.00	32.12

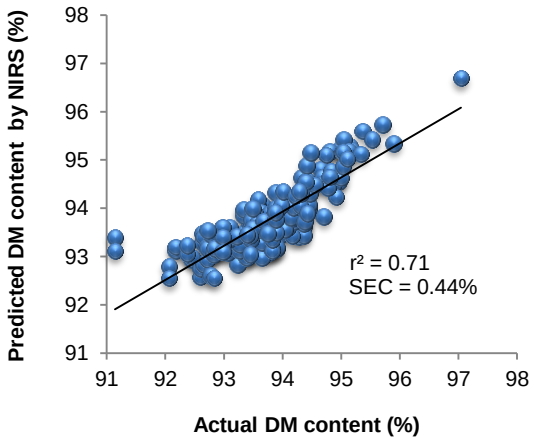
A useful value for evaluation of a calibration involves the ratio of the SEP to the SD statistic, which is termed the RPD. It is the ratio of the standard error of prediction to standard deviation of the reference data of the validation sample set. The SEP should be much lower than the SD and the ideal ratio should be 5 or more, but at least 3 (Williams, 2001). The results for this study indicated RPD values less than 3 for all constituents, which indicate that calibrations are not recommended for predictions by NIR spectroscopy for sunflower seed meal.

Pérez-Vich *et al.* (1998) found that the accuracy and reliability of calibration equations for oil content and individual fatty acids, which were developed from intact seed samples, were lower than those obtained with husked seed, meal and oil. It is not recommended to routinely analyse oil and fatty acid composition in intact sunflower seeds by NIR spectroscopy, because the r^2 values were not high enough which corresponds with

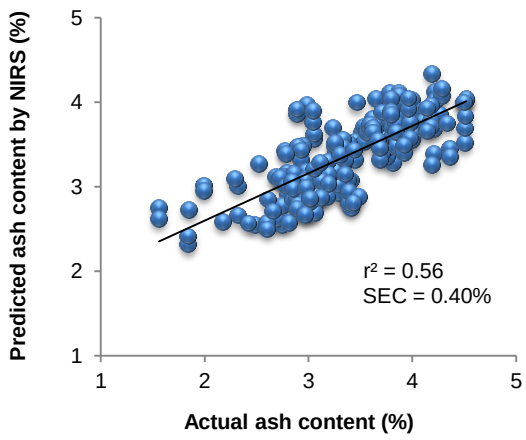
the results found in this study. However, the results revealed that NIR spectroscopy calibration equations for intact seed may be of great use for rapid screening purposes, although it is less accurate than equations for husked seed. Results obtained by Sato *et al*. (1995) reported that the determination of the fatty acid composition of sunflower seeds could be determined by NIR spectroscopy for both extracted oil and kernel seed. As NIR spectroscopy is a non-destructive analysis, the manually husked single-grain seed can still be germinated after the analysis. Pazdernik *et al*. (1997) reported coefficient of determination in validation (r^2_{val}) of 0.38 and 0.18 for ground soybean samples and 0.71 and 0.56 for fatty acids of whole soybean samples. Kovalenko *et al*. (2006) evaluated calibration equations for fatty acid predictions in whole soybeans. The highest predictions were found in total saturated fatty acids (r^2 = 0.91 - 0.94), followed by palmitic acid (r^2 = 0.80 - 0.84), stearic acid (r^2 = 0.49 - 0.68), oleic acid (r^2 = 0.76 - 0.81), linoleic acid (r^2 = 0.73 - 0.76) and linolenic acid (r^2 = 0.67 - 0.74).

The correlation between the laboratory determined values and NIR spectroscopy predicted values and SEP values for dry matter, ash, crude protein, crude fibre and oil content are illustrated in Figure 1.

a)



b)



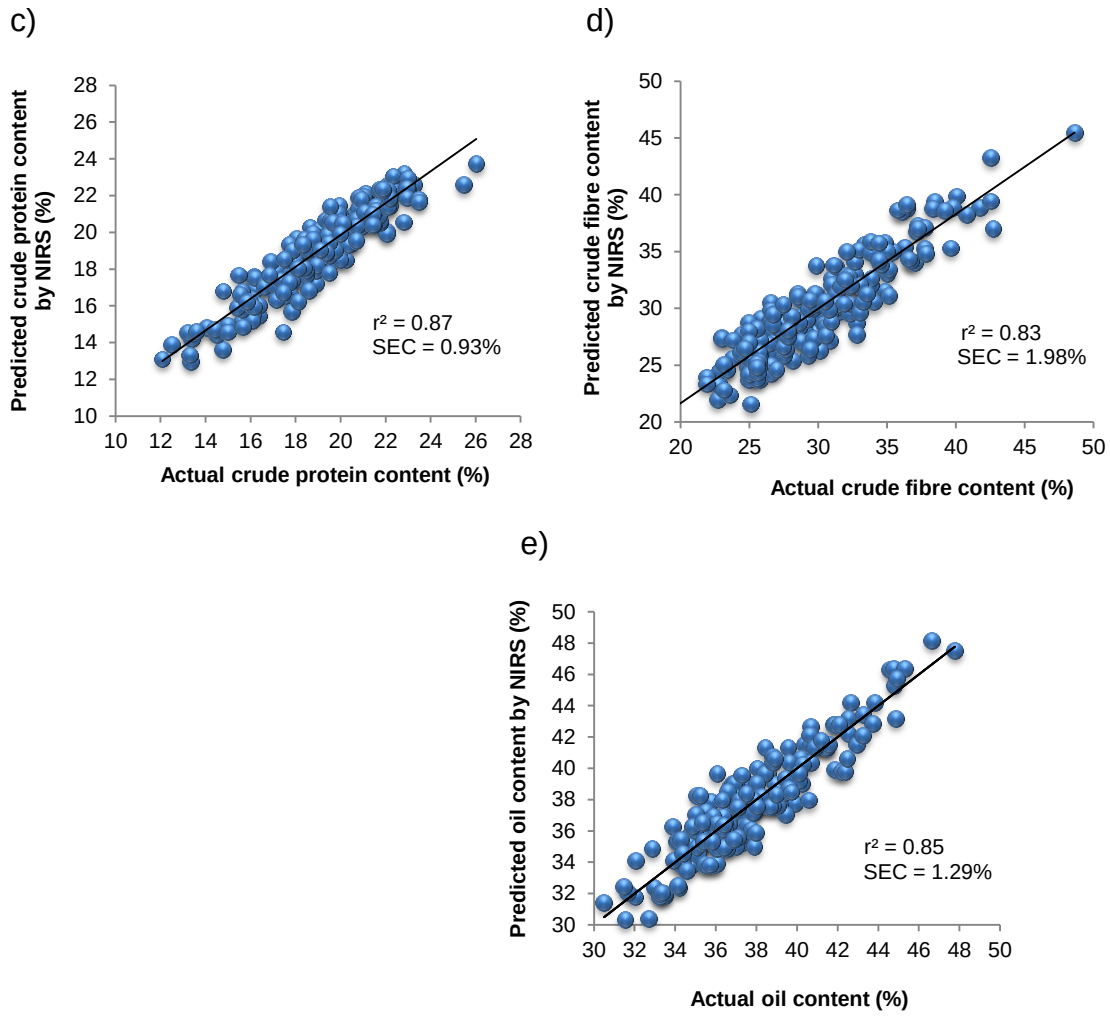


Figure 1 The relationship between laboratory determined and NIR spectroscopy predicted values for (a) DM, (b) ash, (c) crude protein, (d) crude fibre and (e) oil content

The correlation between the laboratory determined values and NIR spectroscopy predicted values and SEP for the individual fatty acids values for palmitic acid, stearic acid, oleic acid and linoleic acid are illustrated in Figure 2. Palmitic acid content (C16:0) of sunflower seed meal was not well predicted (r^2 = 0.40) by NIR spectroscopy. Similarly, NIR spectroscopy did not efficiently predict stearic acid (C18:0) (r^2 = 0.45) in sunflower seed meal.

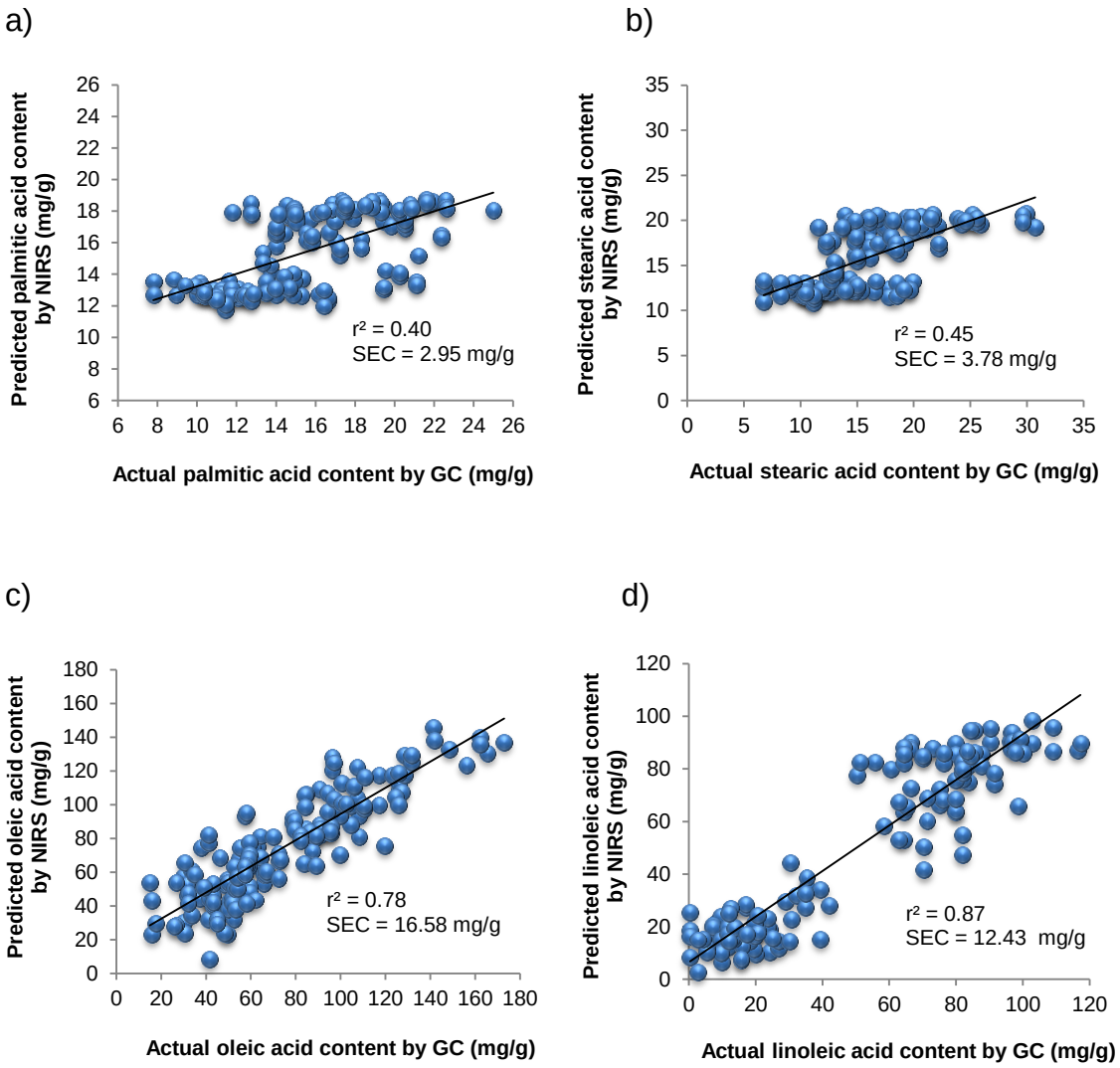


Figure 2 The relationship between laboratory determined and NIRS predicted values for (a) palmitic acid, (b) stearic acid, (c) oleic and (d) linoleic acid

Conclusions

Calibration equations for DM, ash, crude protein, crude fibre, oil content, palmitic acid, stearic acid, oleic acid and linoleic acid were developed on NIR spectroscopy, which shows that the calibrations can be used for screening of sunflower seed samples. Calibration equations for the DM, ash, crude protein, crude fibre and oil content were developed from a set of 160 intact milled sunflower seed samples which were scanned by NIR spectroscopy and further analysed by the reference methods, and 100 samples were used for development of the calibrations for the individual fatty acids. Calibration models for the individual fatty acids, namely palmitic acid, stearic acid, oleic acid and linoleic acid, were scanned by NIR spectroscopy and analysed by gas chromatography (GC).

Mathematical procedures on the spectral information were performed with SESAME Version 2.00 software (Bran & Luebbe GmbH, Norderstedt, Germany). Since NIR spectroscopy predictions are faster and more cost-effective than GC analysis, NIR spectroscopy can be used alternatively to GC to speed up selection programmes. Furthermore, NIR spectroscopy offers the possibility of a non-destructive analysis of the fatty acid composition of the whole seed in materials with non-uniform fatty acid composition along the seed, for which GC cannot be used.

SEC values ranged from 0.40% for ash to 1.98% for crude fibre and r^2 values ranged from 0.56 for ash to 0.87 for crude protein. In the validation tests the coefficient of determination in calibration (r^2_{val}) values range from 0.53 for crude fibre to 0.83 for crude protein and SEP values ranged from 0.50% for DM to 3.39% for crude fibre.

This data suggests that NIR spectroscopy is a rapid tool which can be used for the screening of the nutritional value of fatty acids in sunflower seeds with a precision which can be used as a routine quality control tool. Although the accuracy was too low for routine analysis, NIR spectroscopy could be used as a screening tool to predict the DM, ash, crude protein, crude fibre, and oil content, but was too low for screening of palmitic acid, stearic acid, oleic acid and linoleic acid of sunflower seed meal. The results of this study gives an indication that NIR spectroscopy could be successfully used for the prediction of chemical composition and fatty acid concentration if a large enough calibration set was used to develop the calibration. Screening of sunflower seeds by NIR spectroscopy can represent a rapid, simple and cost effective alternative that is a great utility for users who need to analyse a large number of samples. For the successful development of accurate calibration models to estimate the fatty acid composition in sunflower seed samples by NIR spectroscopy, a calibration with large variability for fatty acid composition and good repeatability in the analyses by the reference method is needed.

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CHAPTER 3

THE USE OF NEAR INFRARED (NIR) SPECTROSCOPY TO PREDICT THE CHEMICAL COMPOSITION OF MILLED AND WHOLE CANOLA SEED (*BRASSICA NAPUS* L.)

Abstract

The potential of near infrared reflectance (NIR) spectroscopy to determine the chemical composition of milled and whole canola seeds (*Brassica napus* L.) was investigated. Standard quality analyses, like ether extraction for oil content, are laborious and time-consuming, which is why a fast method for standard quality analyses is desirable. A non-destructive method for the screening of seed samples would also allow high throughput analyses in plant breeding and genotype selection. For this study, 138 canola seed samples were analysed for DM, crude protein and oil content by traditional wet chemistry analysis. The samples were scanned on a NIR spectroscopy model Perten DA7200 and calibration equations were developed for each nutrient in both milled and whole seed samples. The calibration was focused on the possibility of screening whole seed samples of different compositions of DM, crude protein, and oil content using NIR spectroscopy analysis, which would save a considerable amount of time as milling of samples is laborious and time consuming. The verification of a validation equation in 27 randomly selected samples in milled canola seed samples, proved high coefficients of determination (r^2) between NIR spectroscopy analysis and laboratory reference values for crude protein (0.91) and oil content (0.91) although not as accurate for DM (0.57). The standard deviation (SD) to standard error of prediction ratio (RPD) values for DM was 1.53, 3.18 for crude protein and 3.09 for oil content. The corresponding results for whole canola seeds were crude protein (r^2 = 0.80) and oil content (r^2 = 0.86), but not as accurate for DM (r^2 = 0.57). The RPD was 1.52 for DM, 2.25 for crude protein and 2.61 for oil content. Although the results indicated that calibrations were better for milled canola seeds, it indicated values that were typical of equations suitable for screening purposes to select samples for more detailed chemical analysis.

Keywords: intact canola seed, milled canola seed, nutritive value, NIR spectroscopy

Introduction

Canola and rapeseed is the second most important oilseed crop in the world and currently contributes close to 14% of the total world production of oilseeds with a total world production over the past three years of 63.9 million tons annually (PRF, 2013) and 112 000 tons produced in South Africa (Dredge, 2014).

Canola is a feed ingredient in animal nutrition which can be included in complete diets of a wide range of livestock with great benefits as either full-fat seed (the unprocessed seed) or as protein-rich residue canola oilcake meal (the residue that is left once the oil has been removed from the seed). The oil content of full-fat canola is in the range of 36 - 50% and its crude protein content about 20 - 25%, whereas the canola oilcake contains nearly 37% protein. The optimum inclusion level of full-fat canola is approximately 12% in the complete diets of lambs and 6% in the complete diets of dairy cows. The ideal inclusion level of full fat canola in the diet of monogastric animals ranges from 12 - 18% for pigs, 10% for ostriches and the maximum inclusion level for chickens is 5 - 10%. The inclusion of full-fat canola in the diets of chickens, pigs and dairy cows results in healthier fat and milk fat profiles, since canola contains a higher concentration of unsaturated fats versus saturated fats (de Kock & Agenbag, 2009). The good amino acid ratio, a relatively low apparent metabolisable energy (AME) and high levels of phytate (3%), provide benefits for inclusion in poultry diets (Classen *et al*., 2004).

The use of fast analytical techniques, such as NIR spectroscopy, has many advantages compared with standard analytical techniques. NIR spectroscopy analysis is carried out with considerable saving of time and cost and operates without using hazardous chemicals. In addition, NIR spectroscopy is a non-destructrive technique and samples can be analysed in their natural form without destruction (Font *et al*., 2006). The technique is being increasingly utilised nowadays and can be used for the simultanous evaluation of several components, such as the estimation of oil, protein, total glucosinolates and other characteristics in routine analysis of whole canola seed samples (Prem *et al*., 2012), as well as for other seed quality parameters, which are highly desirable in plant breeding (Rudolphi *et al*., 2012). This is due to the relative ease of sample preparation and the flexibility of sample presentation. The technology is now utilized extensively throughout the world at grain receiving points, commercial oil producers and analytical laboratories, particularly for the evaluation of oil and moisture content in intact seed. With the use of advanced spectrometrics and chemometrics

software, the selection of calibration samples is based on relevant spectral information; this is important when establishing robust calibrations. There is still, however, a need to conduct primary analyses on samples for the development, validation and maintenance of calibrations (Pallot & Golebiowski, 2014). Plant breeding programmes usually involve extensive evaluations of the quality components of seeds. Thus, large numbers of screenings by standard analytical methods of seed lines are usually performed, in order to detect target genotypes. Although standard analytical techniques usually offer a high level of accuracy and precision, they also show some disadvantages, such as high costs, high labour input and delay in reporting. In addition, many standard techniques involve the destruction of the test sample, which could have a negative impact in the case of valuable and scarce materials (Font *et al*., 2006).

NIR spectroscopy is an analytical technique which measures light absorption of radiation in the region of 400 - 2500 nm (visible plus near-infrared regions) which is closely related to important chemical bonds (O-H, N-H and C-H) and is used to develop calibration curves, which can be related to chemical composition in the sample. After calibration, the regression equations developed allows accurate analysis of many other samples by prediction of data based on the spectra (Font *et al*., 2006). The application of NIR spectroscopy technology allows established knowledge of the science of animal nutrition to be readily and objectively applied to improve productivity and cost-effectiveness of livestock production systems. In the animal feed manufacturing industries, NIR spectroscopy can be applied to determine important nutrient profiles of concentrates and forage feeds used as ingredients, and for quality control during manufacturing of products for both monogastric and ruminant animals. It thus allows quality control and application of nutritional science in animal feed manufacturing and, consequently, animal production systems, to an extent not previously practicable (Dixon & Coates, 2010).

This study was conducted to develop NIR spectroscopy equations to predict the chemical composition of milled and whole canola seeds. The development of calibration equations on the samples chosen for this study were done on canola seed samples from different seasons with significant sample variation and could produce a more robust calibration to determine moisture, crude protein, and oil content. Traditional wet chemical analysis was used to determine the nutritive composition of canola seeds, but these procedures are costly, time-consuming and sometimes hazardous.

The aim of this study was therefore to determine the suitability of a NIR spectroscopy instrument for the effective screening selection of whole canola seed to predict dry matter, crude protein, and oil content in whole canola seeds compared to milled canola seeds, with different nutrient content for utilization in the Swartland and Southern Cape breeding programmes in South Africa.

Materials and methods

Sampling and preparation

A total of 138 canola seed samples were collected from several experimental farms in the Swartland and Southern Cape regions of South Africa, which varies in location, soil characteristics (texture, organic matter, nitrogen content, pH) and farm management. The samples were used to develop NIR spectroscopy calibration equations in milled and whole canola seeds. The samples consisted of different cultivars of canola, including Agamax, Jardee, Thunder, and Cobler.

Sample preparation for conducting the reference analysis included milling the samples for 2 x 10 seconds intervals per sample at 20000 rpm by making use of a Foss Tecator Knifetec 1095 sample mill, equipped with a rotor blade with sharp knives (FOSS Analytical, Hilleröd, Denmark). The Knifetec 1095 sample mill is designed for the preparation of high fat, high moisture and/or fibrous samples prior to analysis and therefore is the ideal mill for grinding of canola seeds. The mill is equipped with a grinding chamber cooling feature which enables it to be connected to a cold water tap to reduce adhesion of high oil samples to the wall of the grinder. Samples containing high levels of fat have the tendency to stick to the wall of the chamber as the fat softens during grinding, preventing adequate homogenisation (Foss Analytical, 2005).

Analyses of reference samples

Dry matter (DM), crude protein (CP) and oil content analysis was performed on 138 milled canola seed samples. The nutritive value of canola seeds was determined using official methods as described by the Association of Official Analytical Chemists (AOAC, 2012). Moisture content was determined by placing 2 g of milled canola seed in a forced-air oven at 100 °C for 24 hours, by using the AOAC (2012) (Method no: 934.01). The crude protein content was estimated by determining the nitrogen content by the combustion method (AOAC, 2012) (Method no: 990.03) using a LECO TruMac N Nitrogen

Determinator, Version 1.3X (LECO Corporation, St. Joseph, Michigan, USA) and subsequent multiplication of the nitrogen content value by 6.25, the universally accepted protein content estimation factor. Oil content was determined by solvent extraction on a Soxtec system HT 1043 (Tecator, Höganas, Sweden) by using diethyl ether as extraction fluid (AOAC, 2012) (Method no: 2003.06). All chemical analysis results are expressed on an as is basis and samples were analysed in duplicate.

Collection of spectra and calibration development

In total, 138 canola seed samples representing a wide range of chemical characteristics, from different years, cultivars and locations, were scanned as whole seeds as well as milled seeds in an NIR spectrometer instrument. The canola seed samples were scanned as duplicates in the reflectance mode between 950 - 1650 nm and recorded as log (1/R) at 2nm increments of the near-infrared region on a Perten DA7200 Diode Array analyser (Perten Instruments AB, Huddinge, Sweden). Approximately 80 g of each sample in whole seed form and 50 g of each milled seed sample was packed into an open rotating sample cup with a diameter of 75 mm. Subsequently, the sample set was split into two sets: the larger set was used as the calibration set (n = 111) for development of the calibrations and the smaller set was used as the external validation set (n = 27) and was used to test the accuracy of the calibrations. The samples were divided in such a way that samples from the different varieties were contained in both sets (Perten, 2007).

Partial least squares (PLS) regression was applied to obtain mathematical models comparing the spectral data and the reference laboratory data. Models were built by full cross validation by using Unscrambler version 10.3 (Camo, Trondheim, Norway) software. NIR spectroscopy is widely used as a quantitative method and the main multivariate techniques consist of regression methods used to build prediction models (Dardenne *et al*., 2000). Modified partial least squares regression was used, as it can deal with non-linearity and produce accurate models for the analysis of agricultural products (Dardenne *et al*., 2000).

After elimination of outliers, all calibrations were developed using full spectrum information and different mathematical treatments were tested by correcting the original reflectance spectra prior to calibration. This was done by applying Smoothing Savitzky Golay, 2^{nd} derivative transformation with Savitzky Golay (2^{nd} order polynomial, 7 point smoothing), standard normal variate (SNV) and SNV with de-trend scatter correction

(polynomial order = 2). The most appropriate models were chosen by the highest coefficient of determination (r^2) and lowest standard error of calibration (SEC). For milled canola seeds the Savitzky Golay 2nd derivative was applied for DM and oil content and for crude protein calibration SNV was applied. Pre-processing used for whole canola seeds included Savitzky Golay 2nd derivative transformation for development of DM, crude protein and oil content calibrations.

The original (a), pre-treated Savitzky Golay 2nd derivative (b) and SNV (c) spectra of milled canola seed samples are shown in Figure 1 and original (a) and Savitzky Golay 2nd derivative data (b) obtained from canola whole seed samples are demonstrated in Figure 2.

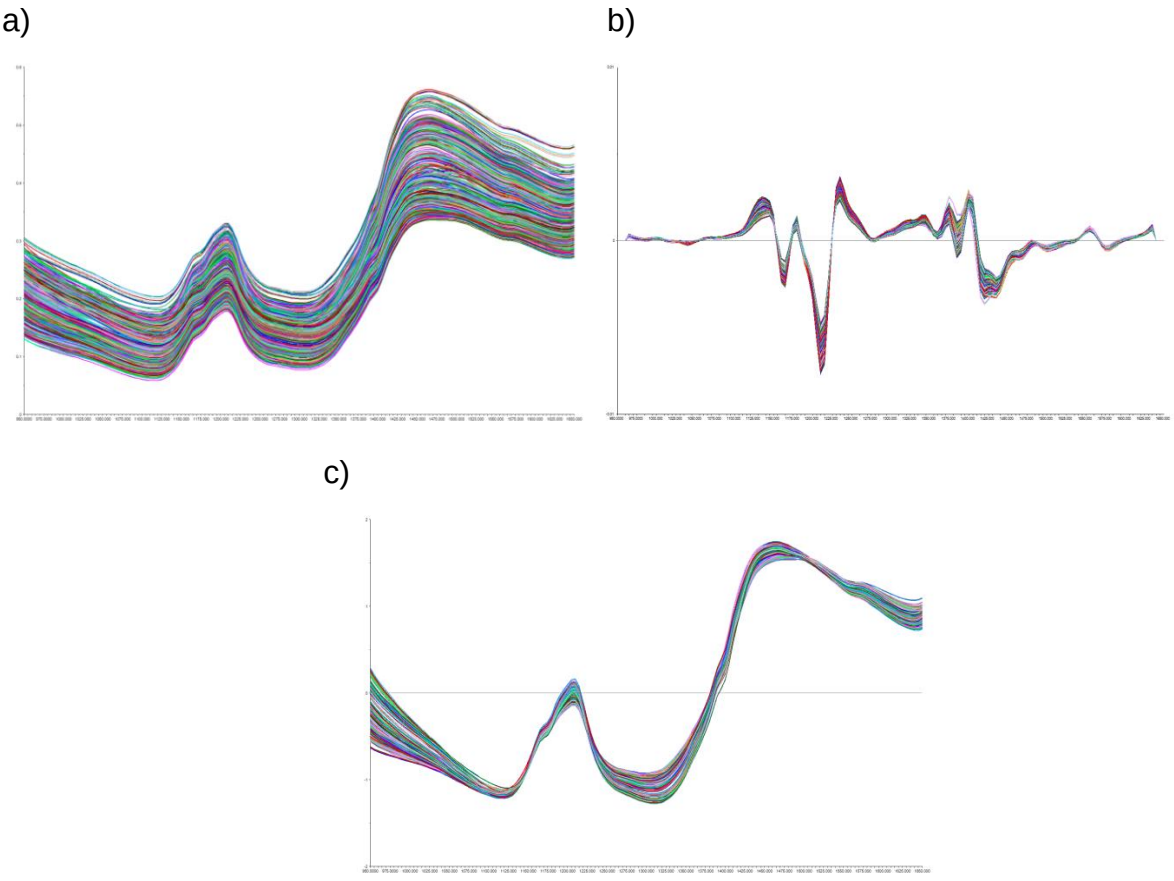


Figure 1 Absorbance spectra for milled canola seeds from (a) raw data, (b) Savitzky Golay 2nd derivative data and (c) standard normal variate (SNV)

a) b)

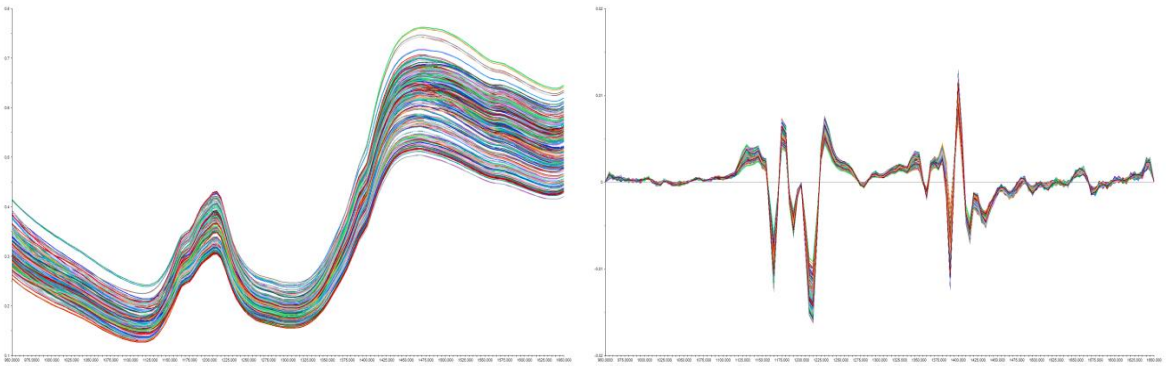


Figure 2 Absorbance spectra for whole canola seeds from (a) raw data and (b) Savitzky Golay 2nd derivative data

The accuracy of the calibration models was expressed by means of the standard error of prediction (SEP), the coefficient of determination (r^2) and the ratio of SEP to standard deviation (SD) of the validation set (RPD), which is an indication of the efficiency of a calibration. The goal of model development is to obtain a calibration model with a low SEP, a high r^2, preferably above 0.83 and a RPD higher than 5. The SEP should also be as close as possible to the standard error of reference data (SEL) (Williams, 2001).

Results and discussion

Calibration equations for rapid analysis by NIR spectroscopy were developed by using partial least square regression (PLS) with cross validation for dry matter, crude protein and oil content in milled and whole canola seed samples. The number of samples (n), minimum, maximum, mean values and standard deviations (SD) for each constituent for the calibration and validation sets of the calibrations developed for milled and whole canola seeds are reported in Table 1 and 2, respectively.

Table 1 Summary of nutrient composition (%) of milled canola seed used in the calibration set, showing the number of samples (n), minimum, maximum, mean and standard deviation (SD)

Chemical component	n	Min (%)	Max (%)	Mean (%)	SD
DM	111	90.66	96.29	94.12	1.01
Crude protein	111	14.14	25.48	21.53	2.17
Oil content	111	34.35	45.90	40.10	3.00

Table 2 Summary of nutrient composition (%) of milled canola seed used in the validation set, showing the number of samples (n), minimum, maximum, mean and standard deviation (SD)

Chemical component	n	Min (%)	Max (%)	Mean (%)	SD
DM	27	89.38	95.92	94.00	1.30
Crude protein	27	16.78	24.59	21.11	2.41
Oil content	27	34.56	46.23	40.74	3.29

Results reported in Table 1 show the reference values used in the calibration set for DM which ranged from 90.66 - 96.29%, crude protein from 14.14 - 25.48% and oil content from 34.35 - 45.90%. Results of the nutrient composition of the samples used in the validation set are reported in Table 2, as the range of DM is 89.38 - 95.92%, crude protein ranges from 16.78 - 24.59% and oil content from 34.56 - 46.23%. Results for reference analysis as reported in Table 1 and Table 2 correspond with ranges obtained by Nosenko *et al*. (2013) for DM of 90 - 92%, Velasco *et al*. (1999) for protein content (13.4% - 28.3%) and oil content (28.5% - 54.9%) and 32.46% to 50.64% as reported by Greenwood *et al*. (1999) for oil content of canola seeds.

The optimum number of PLS factors selected for each calibration were recommended by Unscrambler software using cross validation, as shown in Table 3 for milled canola seed samples and Table 4 for whole canola seed samples. The reliability of calibration models was established based on the values of the coefficient of determination (r^2), standard error of prediction (SEP), standard error of laboratory (SEL) and RPD (the

ratio of standard deviation of reference values in the validation set to SEP) (Williams, 2001). The ratio of performance to deviation (RPD) is a measurement of the ability of an NIR spectroscopy model to predict a constituent. Reporting the SEP alone may be misleading unless it is reported by comparison with the SD of the original reference data. If the SEP is close to the SD, then the NIRS calibration is not efficiently predicting the composition or functionality. If SEP is equal to SD, the calibration is essentially predicting the population mean. Williams (2001) suggested that a RPD value below 2 does not supply accurate predictions. RPD value of 2 - 3 is considered as adequate for rough screening. A value above 3 is regarded as satisfactory for screening, a value of 5 and upward is suitable for quality control analysis and a value above 8 is excellent and can be used in any analytical application.

An external validation procedure (n = 27) was carried out to determine the accuracy and precision of the equations obtained in the calibration for each component in both milled and whole canola seeds. To evaluate the accuracy of the equations, different statistics were used, namely the coefficient of determination (r^2), the RPD, which is the ratio of the standard deviation (SD) for the validation samples to the standard error of prediction (SEP).

Milled canola seeds

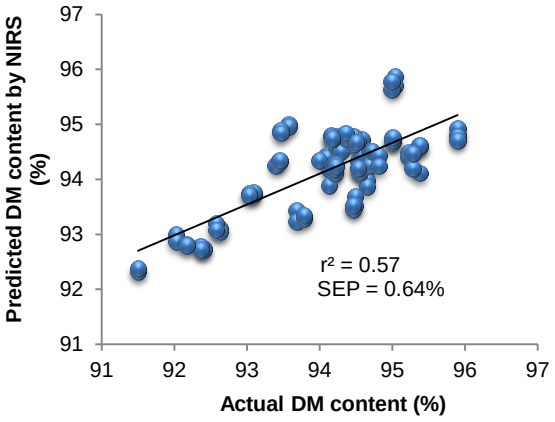
Calibration models were developed by PLS regression from original spectra and after pre-processing, with Savitzky Golay 2^{nd} derivative for DM and oil content and SNV for crude protein. Table 3 displays the results of the multivariate data analysis of the calibrations and their performance on the validation set. The better calibration equation for each constituent, with respect to higher coefficient of determination in calibration (r^2_{cal}), SEC, r^2_{val}, lower SEP, SD and RPD, are shown in Table 3. The calibration model for milled canola seeds is of reasonable quality with an r^2_{val} value in the external validation set and standard error of calibration (SEP) of 0.57 and 0.64% for DM, 0.91 and 0.60% for crude protein and 0.91 and 1.07% for oil content. According to Williams (2001), the RPD values of 1.53 for DM indicate that the calibration is not appropriate for predicting milled canola seeds and a RPD value of 3.18 for crude protein and 3.09 for oil content indicate that the prediction model is satisfactory for screening of milled canola seeds.

Table 3 Calibration and external validation statistics in the development of calibration equations for nutrient composition in milled canola seed samples

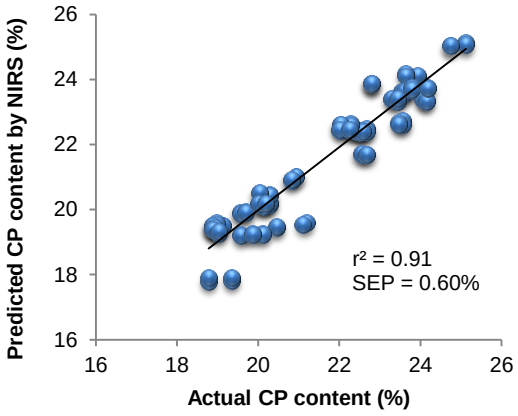
Chemical component	PLS factors	Calibration set		Validation set			
		r^2_{cal}	SEC (%)	r^2_{val}	SEP (%)	SD	RPD
DM	5	0.76	0.54	0.57	0.64	0.98	1.53
Crude protein	7	0.87	0.78	0.91	0.60	1.91	3.18
Oil content	5	0.89	1.00	0.91	1.07	3.31	3.09

The calibration model showed a good agreement between reference and NIR spectroscopy predicted values. As shown in Figure 3 (b) and (c), the high values of r^2_{val} for crude protein (0.91) and oil content (0.91) indicates a good correlation between the predicted and the reference values for the validation model for milled canola seeds. A lower correlation was found for dry matter (r^2_{val} = 0.57) as shown in Figure 3a, which indicates a lower correlation between predicted and reference values for milled canola seeds.

a)



b)



c)

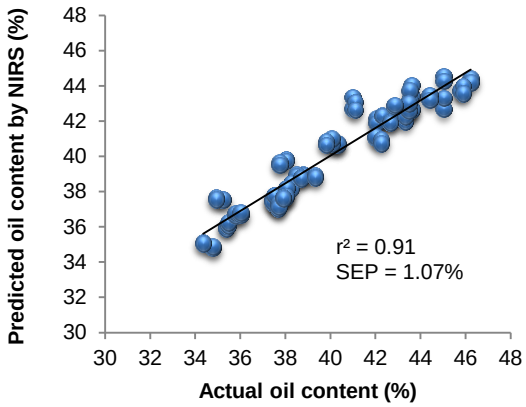


Figure 3 The relationship between laboratory determined and NIR spectroscopy predicted content for (a) DM, (b) crude protein and (c) oil content in validation statistics for milled canola seed

Whole canola seeds

The NIR spectroscopy calibration and external validation statistics for DM, crude protein and oil content for whole canola seed are presented in Table 4. The calibration equation for DM did not show adequate validation statistics, as the coefficient of determination (r^2_{val}) between NIR spectroscopy and reference data was low (r^2 = 0.57) and the ratio of the standard deviation of the calibration set to the standard error of prediction to the (RPD) was 1.57. The calibration equation for crude protein was more accurate, exhibiting an r^2 of 0.80 and a RPD value of 2.27 and the values for oil content was 0.86 and 2.61, respectively. The standard error of prediction (SEP) for the NIR spectroscopy calibration models for intact canola seeds were as follows: dry matter (SEP: 0.83%), crude protein (SEP: 1.06%) and oil content (SEP: 1.26%).

However, it is suggested that the technique could be used as a routine procedure to apply in breeding programmes, only if calibration is done for each species, season and particular conditions (Garcia & Cozzolino, 2006). Additionally, NIR spectroscopy calibrations were more accurate for chemical composition of milled seed than whole canola seeds. In previous studies done by Pazdernick *et al*. (1997) and Pérez-Vich *et al*. (1998) the same relationship was observed in soybean and sunflower seeds respectively, where better calibration equations were obtained for ground seed than whole seed samples.

Table 4 Calibration and external validation statistics in the development of calibration equations for nutrient composition in whole canola seed samples

Chemical component	PLS factors	Calibration set		Validation set			
		r^2_{cal}	SEC (%)	r^2_{val}	SEP (%)	SD	RPD
DM	5	0.86	0.38	0.57	0.83	1.30	1.57
Crude protein	5	0.84	0.86	0.80	1.06	2.41	2.27
Oil content	5	0.92	0.84	0.86	1.26	3.29	2.61

Font *et al*. (2006) obtained RPD and r^2 values of 6.98 and 0.98, respectively, for oil content in *Brassica juncea.* Studies done by Velasco *et al*. (1999), revealed that NIR spectroscopy calibration models were developed to simultaneously predict the oil, protein and glucosinolate contents and oleic acid and erucic acid concentrations of single seeds of canola, which is a non-destructive, fast and cost-effective method. Reliable calibration equations were developed for oil (r^2 = 0.87 and SECV = 1.90%), crude protein (r^2 = 0.91 and SECV = 0.94%) in cross validation and can thus be used for screening of single seeds for quality characteristics in canola. Prem *et al*. (2012) obtained calibration statistics for three *Brassica* species for oil content (SECV = 1.30, r^2 = 0.94, SEC = 1.18), moisture content (SECV = 0.12, r^2 = 0.87, SEC = 0.39) and protein content (SECV = 12.19, r^2 = 0.91, SEC = 2.18). Greenwood *et al*. (1999) have reported on the development of oil content estimation calibrations in canola whole seed (SECV = 0.77, SEC = 0.61 and r^2 = 0.98). The potential of NIR spectroscopy to predict the concentrations of various chemical components, or functional properties of plant materials, which is faster and more economical than conventional chemical analysis, should be a challenge to analysts. If the costs are sufficiently lower, then monitoring, rather than once per season analysis by making use of NIR spectroscopy, could be extremely informative, even if the calibrations have a high SEP or low RPD value (Batten, 1998).

The correlation between the NIR spectroscopy predicted and laboratory determined values of the validation sets for the various chemical components found in canola seeds are illustrated in Figure 4 (a) for DM, (b) for crude protein and (c) for oil content in whole canola seeds.

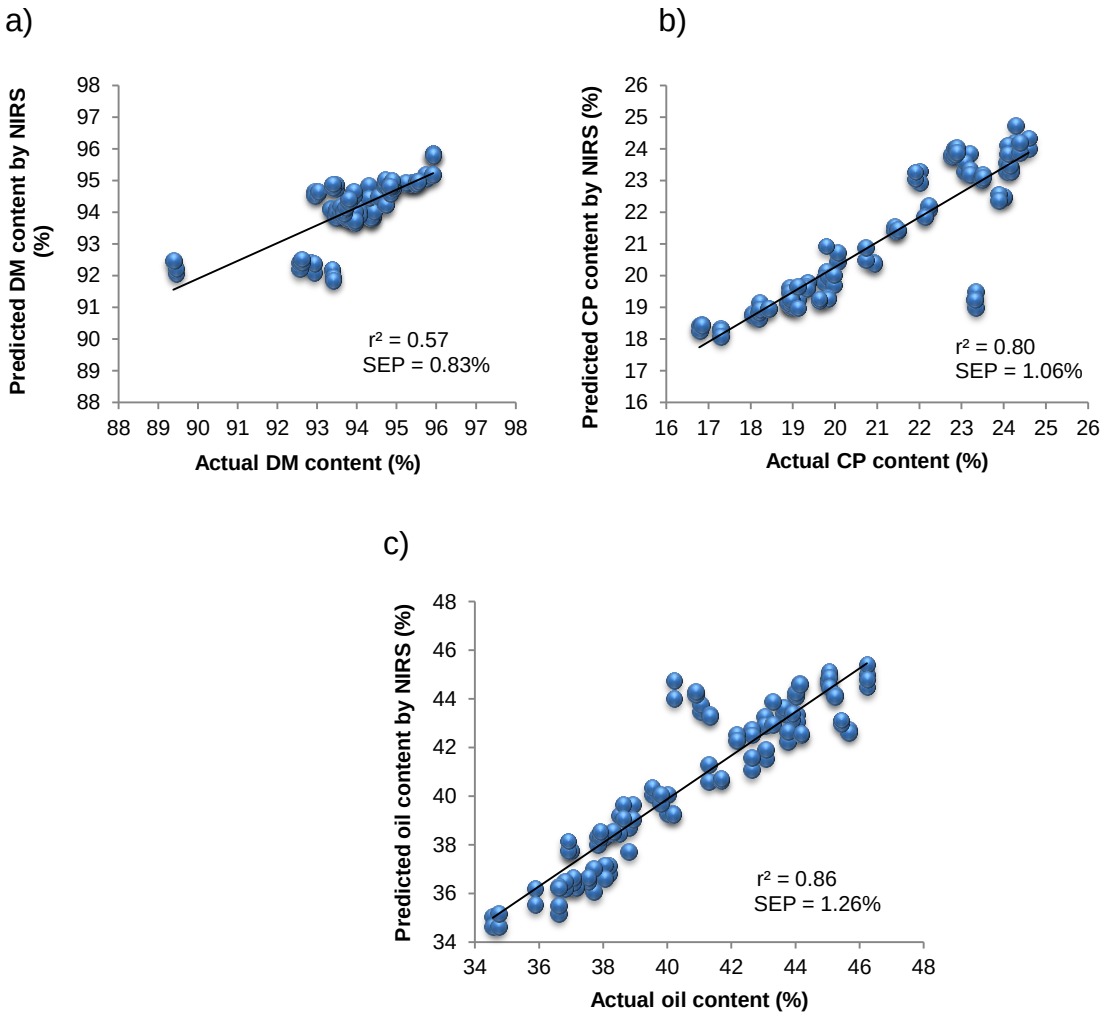


Figure 4 The relationship between laboratory determined and NIR spectroscopy predicted content for (a) DM, (b) crude protein and (c) oil content in validation statistics for whole canola seed

Conclusions

The chemical composition of canola, predicted by NIR spectroscopy was highly correlated with values determined by AOAC analytical methods. The results demonstrate the potential of NIR spectroscopy to predict the chemical composition of different canola cultivars. This indicates that NIR spectroscopy can be used as a reliable tool in the screening process of milled and whole canola seeds. This will result in a rapid, less expensive analysis technique, which will benefit the feed industry in formulations of balanced diets. The accuracy was quite satisfactory for screening purposes as RPD values was between 2 and 3 for crude protein and oil content for whole canola seeds and above 3 for milled canola seeds. The analysis of whole canola seeds by NIR spectroscopy

was less accurate (r^2 ranged from 0.57 for DM to 0.86 for oil content) although it is reliable enough to use for pre-screening purposes to identify variants with significantly different nutritive values. Non-destructive NIR spectroscopy analysis enables rapid and reliable selection of materials with different nutrient composition in whole canola seed. It can be concluded that NIR spectroscopy can be used reliably for non-destructive selection of chemical components in both milled and whole canola seeds.

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CHAPTER 4

PREDICTION OF THE CHEMICAL COMPOSITION OF MILLED AND WHOLE LUPIN SEEDS WITH NEAR INFRARED REFLECTANCE (NIR) SPECTROSCOPY

Abstract

The potential of near-infrared reflectance (NIR) spectroscopy to perform an easy and rapid estimation of dry matter (DM), ash, crude protein (CP), crude fibre and oil content in milled and whole lupin seeds was investigated. Lupin samples from different cultivars produced at different localities in the Western Cape regions of South Africa were collected. A total of 160 samples were used to develop calibrations for milled lupin seeds and 49 to develop calibrations for whole lupin seeds. All samples were analysed by traditional wet chemistry techniques for DM, ash, crude protein, crude fibre and oil content and were subsequently scanned on a NIR spectroscopy model Perten DA7200. Calibration equations for the different chemical components were developed for each nutrient in both milled and whole lupin seed. For both milled and whole lupin seed, the performance of the calibration equations was evaluated through external validation. The results showed that NIR spectroscopy is a reliable and accurate technique to estimate these constituents in whole lupin seeds and milled lupin seeds. The validation statistics revealed better results for whole lupin seed than milled lupin seed. The validation r^2 for milled lupin seeds ranged from 0.55 for crude fibre to 0.85 for oil content, whereas the r^2 in whole lupin seeds ranged from 0.66 for ash to 0.96 for crude protein. NIR spectroscopy equations showed RPD values of 1.39 to 2.52 for milled lupin seeds and 1.65 to 4.76 in whole lupin seeds that were indicative of equations suitable for screening. According to these results, there is no need to grind the seeds to scan the meal; similarly accurate results are obtained by analysing whole seeds. Screening of whole lupin seeds by NIR spectroscopy represents a rapid, simple and cost effective alternative that may be of great utility for users who need to analyse a large number of samples with no sample preparation.

Keywords: lupin whole seeds, chemical composition, NIR spectroscopy

Introduction

Lupins are the harvested seed of species from the *Lupinus* genus, a group within the leguminous bean and pea family *Fabaceae,* and are known as a rich source of protein which has long been used in human and animal nutrition. The major cultivated species of lupins are: *L. angustifolius* (narrow-leafed sweet lupin), *L. albus* (white or albus lupin) and *L. luteus* (yellow lupin), where *L. angustifolius* dominates world lupin production (Glencross, 2007). Lupins play an important role as an alternative crop in the Western Cape and approximately 18 000 ha lupins are currently cultivated in the Western Cape winter rainfall area (Dunn, 2015).

Lupin seed has the potential to be a vegetable protein source due to its comparable quality to the commonly used soy proteins (Bartkiene *et al*., 2011). Lupin grain is considered as a valuable protein source in monogastric (Brand *et al*., 1995) and ruminant animals (Brand *et al*., 1997) as well as in aquaculture nutrition, such as salmon (Alomar & Mera, 2008) and rainbow trout (Glencross *et al*., 2015). The evaluation of NIR spectroscopy in agricultural applications started when Karl Norris applied the statistical regression data analysis method in NIR diffuse reflectance studies in the 1960's (Norris & Hart, 1965). Since then, many NIR spectroscopy related applications have been reported for oil seed analysis. NIR spectroscopy is a rapid, non-destructive, inexpensive and accurate method for the analysis of chemical components and material characteristics in seeds, grains and other types of materials. Another advantage of modern NIR spectroscopy instruments is its capability of producing multiple results from one single analysis of intact samples (Tseng *et al*., 2004).

Analysis of nutrient composition of oil seeds plays an important role in the quality control and assurance of oil seeds in both agriculture and food industries. Conventional analytical methods of oil seeds are often time consuming, labour intensive and expensive. Different analytical methods are required for each oil seed parameter or trait of interest and the analysis of each method individually is time consuming and can last hours or days (Tseng *et al*., 2004).

NIR spectroscopy depends on calibrations which use absorbances at many wavelengths, to predict the composition of a sample. The greatest advantage of the NIR spectroscopy technique is that minimal sample preparation is required and the speed of analysis. Compared to conventional analytical techniques which, however, involves high costs and labour requirements are excessive. These advantages make it possible to

analyse large batches of samples in a short period of time (Batten, 1998). Unlike most conventional analytical methods, NIR spectroscopy is a non-destructive technique, which requires little or no sample preparation, does not use chemicals, or generate chemical wastes which requires disposal. The technique is entirely safe to operate, rapid, can be portable, and simultaneously determines numerous constituents or parameters. NIR spectroscopy instrumentation is simple to operate by non-chemists and operates without fume hoods, drains or other installations (Williams, 2012).

Accurate calibration equations have been obtained by several authors for predicting chemical composition, digestibilities and anti-nutritional factors in lupin seed. Glencross *et al*. (2015) obtained accurate calibrations for DM, ash, crude protein, crude fibre, oil content, acid detergent fibre (ADF), neutral detergent fibre (NDF), lignin, digestible protein and digestible energy. Viljoen (2003) developed accurate calibrations for DM, ash, crude protein, oil content, ADF, NDF, *in vitro* organic matter digestibility (IVOMD), total digestible nutrients (TDN), and accurate calibrations for alkaloids in milled lupin seeds were obtained by Brand & Brandt (2000). Results obtained by Alomar & Mera (2008) showed the remarkable potential of the NIR spectroscopy technique for selecting lupin individuals with lower seed coat in whole grains, indicating that this could be a valuable tool in breeding programmes oriented to improve the feeding quality of lupin grains.

The objective of this study was to develop NIR spectroscopy calibrations and to explore the technique to be used as a rapid technique to estimate the dry matter (DM), ash, crude protein (CP), crude fibre (CF) and oil content in whole lupin seeds compared to milled seeds, which will save a considerable amount of time by not grinding the samples.

Materials and methods

Sampling and preparation

Lupin samples were obtained from different lupin cultivars produced at different localities in the Western Cape region of South Africa. A total of 160 narrow leaf lupins (*Lupinus angustifolius*) samples were analysed to develop calibrations for DM, ash, crude protein, crude fibre and oil content for milled lupin seeds and 49 samples were used for developing calibrations for whole lupin seeds. Samples were ground using a Retsch^{TM} ZM200 sample mill (Haan, Germany) with a 1.5 mm screen to create consistent particle size meal.

Analysis of reference samples

During this study, the nutritive value of lupin seeds was analysed by making use of official methods as described by the Association of Official Analytical (AOAC, 2012). The lupin seed samples were analysed for dry matter (DM), ash, crude protein (CP), crude fibre (CF) and oil content. The DM of each sample was calculated by gravimetric analysis following oven drying at 100 °C for 24 hours (AOAC, 2012) (Method no: 934.01). Ash content of each sample was determined following the loss of mass after combustion of the sample in a muffle furnace (Labcon Muffle furnace RM7) at 550 °C for 8 hours (AOAC, 2012) (Method no: 942.05). Nitrogen concentration was determined by the Dumas combustion method (AOAC, 2012) (Method no: 990.03) using a LECO TruMac N Nitrogen Determinator, Version 1.3X (LECO Corporation, St. Joseph, Michigan, USA). Nitrogen content was then transformed by the factor 6.25 into crude protein content. Crude fibre content was determined by using the method of Goering & van Soest (1970) making use of FIWE Raw Fiber Extractor, Velp Scientifica (Velp Scientifica, Milano, Italy). Oil content was determined by solvent extraction on a Soxtec system HT 1043 (Tecator, Höganas, Sweden) by using diethyl ether as extraction fluid (AOAC, 2012) (Method no: 2003.06).

All chemical analysis were performed in duplicate and results are expressed on an as is basis.

Collection of spectra and calibration development

The lupin seed samples were scanned by making use of Diode Array Near Infrared Spectrometer DA7200 (Perten Instruments, Huddinge, Sweden). The samples were scanned in reflectance mode using a rotating 75 mm cup. The spectra were collected from the whole lupin seeds, as well as from milled lupin seed samples, across the full wavelength range (950 - 1650 mm) of the instrument as absorbance at a resolution of 2 nm using nine scans per sample (Perten, 2007). The samples were scanned in duplicate and were repacked as indicated by the instrument software. The reference data was incorporated in the Unscrambler software 10.3 (Camo, Trondheim, Norway) and the spectra were linked with the nutrient composition of each individual sample.

The calibration equations were calculated using partial least squares (PLS) regression and cross validation technique using Unscrambler software 10.3. Cross validation was used to evaluate the relationship between the spectra and the nutrient composition of the milled and whole lupin seeds. The equations with the lowest standard

error of calibration (SEC) and highest coefficient of determination (r^2) were used to develop calibrations after different mathematical pre-treatments were followed. The pre-processing methods included Smoothing Savitzky Golay, 2nd derivative transformation with Savitzky Golay (2nd order polynomial, 7 point smoothing), standard normal variate (SNV) and SNV with de-trend scatter correction with polynomial order = 2. A total of 38 randomly selected samples were reserved for external validation of milled lupin seeds, and 12 for whole lupin seeds. NIR spectroscopy calibration equations for DM, crude protein, crude fibre and oil content were developed by using the remaining 160 samples for milled seed calibrations and 49 samples for whole seed calibrations.

The original spectra obtained from scanning the milled lupin seeds are shown in Figure 1a, and corrected spectra by applying standard normal variate (SNV) and detrend pre-processing techniques, as shown in Figure 1b. Original reflectance spectra were corrected before calibration by applying standard normal variate (SNV) for development of DM, ash, crude protein and oil content calibrations and gap derivative for crude fibre calibrations in lupin whole seeds. Original reflectance spectra for whole lupin seeds are illustrated in Figure 2a, SNV data in Figure 2b and gap derivative data in Figure 2c.

a) b)

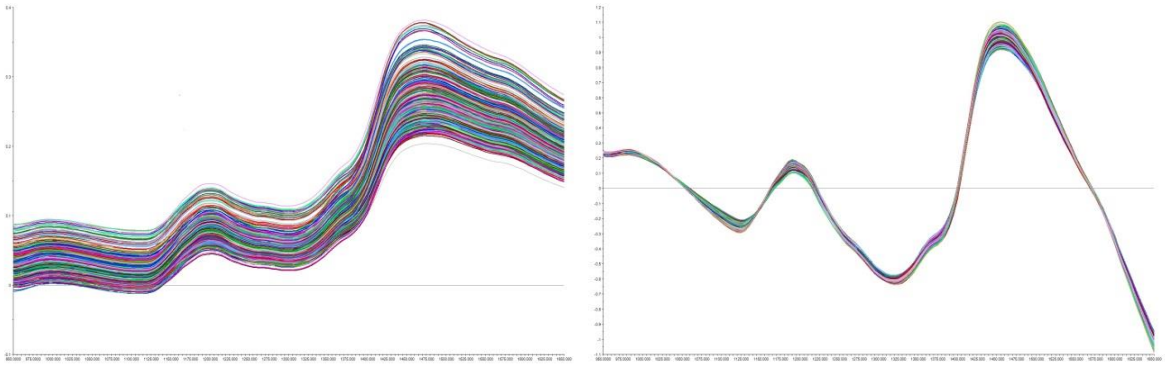


Figure 1 Absorbance spectra for milled lupin seed for (a) raw data and (b) standard normal variate (SNV) detrend data in the NIR spectroscopy range

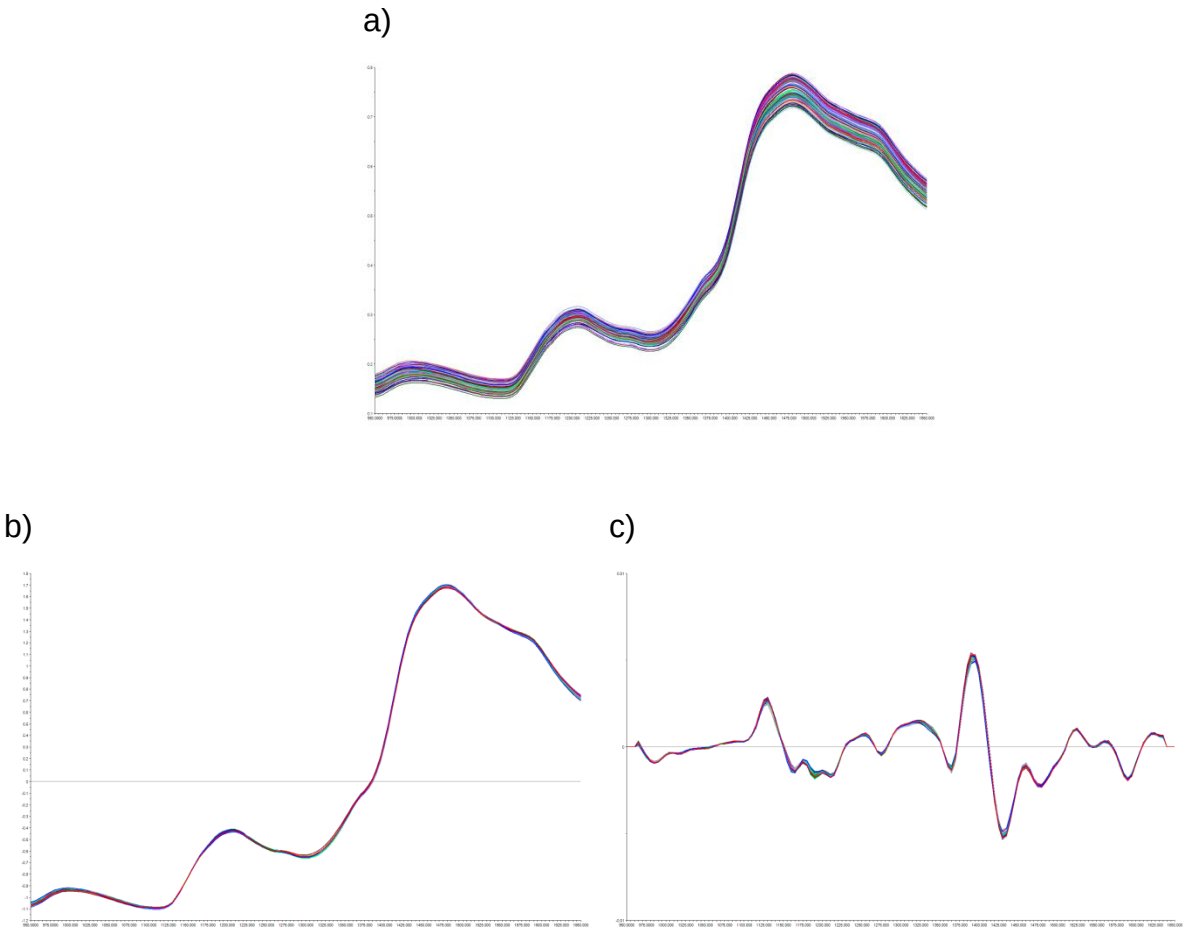


Figure 2 Absorbance spectra for whole lupin seed for (a) raw data, (b) standard normal variate (SNV) data and (c) gap derivative data in the NIR spectral range

Results and discussion

The nutrient composition of lupin seeds used in developing calibrations and validating the calibration in milled lupin seeds are presented in Table 1 and 2, respectively. The number of samples (n), range, mean and standard deviation (SD) values, was calculated for milled lupin seeds and results for the calibration and validation sets are reported in Table 1 and 2, respectively.

Table 1 Summary of nutrient composition (%) of milled lupin seed used in the calibration set, showing the number of samples (n), minimum, maximum, mean and standard deviation (SD)

Chemical component	n	Min (%)	Max (%)	Mean (%)	SD
DM	156	87.08	91.01	89.94	0.86
Ash	160	2.02	3.53	2.89	0.27
Crude protein	160	21.00	34.63	29.24	2.43
Crude fibre	160	12.43	18.70	15.71	1.40
Oil content	160	3.41	6.58	4.95	0.70

Table 2 Summary of nutrient composition (%) of milled lupin seed used in the validation set, showing the number of samples (n), minimum, maximum, mean and standard deviation (SD)

Chemical component	n	Min (%)	Max (%)	Mean (%)	SD
DM	38	86.47	92.11	89.67	1.14
Ash	38	2.41	3.53	2.89	0.28
Crude protein	38	25.75	32.81	29.06	1.90
Crude fibre	38	13.11	18.87	15.76	1.56
Oil content	38	3.57	6.09	4.94	0.68

Results reported in Table 1 show the reference values in the calibration set for DM which ranged from 87.08 - 91.01%, ash from 2.02 - 3.53%, crude protein from 21.00 - 34.63%, crude fibre from 12.43 - 18.70% and oil content from 3.41 - 6.58%. Results of the nutrient composition of the samples used in the validation set are reported in Table 2 with the range for DM as 86.47 - 92.11%, ash from 2.41 - 3.53%, crude protein ranges from 25.75 - 32.81%, crude fibre from 13.11 - 18.87% and oil content from 3.57 - 6.09%.

The nutrient composition of lupin seeds used in developing calibrations in whole seeds is presented in Table 3 and 4. The number of samples (n), range, mean and standard deviation (SD) values were calculated for lupin seeds used in the development of calibrations for whole seeds and results for the calibration and validation sets are reported in Table 3 and 4, respectively. Results reported in Table 3 show the reference values in

the calibration set for DM which ranged from 87.08 - 89.82%, ash from 2.58 - 2.93%, crude protein from 21.00 - 31.13%, crude fibre from 13.80 - 17.48% and oil content from 4.39 - 5.66%. Results of the nutrient composition of the samples used in the validation set are reported in Table 4 with the range for DM as 87.08 - 89.37%, ash from 2.63 - 3.00%, crude protein ranges from 21.00 - 31.44%, crude fibre from 14.29 - 17.48% and oil content from 4.55 - 5.55%.

Table 3 Summary of nutrient composition (%) of lupin whole seed used in the calibration set, showing the number of samples (n), minimum, maximum, mean and standard deviation (SD)

Chemical component	n	Min (%)	Max (%)	Mean (%)	SD
DM	49	87.08	89.82	88.86	0.61
Ash	49	2.58	2.93	2.78	0.10
Crude protein	49	21.00	31.13	28.30	2.32
Crude fibre	49	13.80	17.48	15.40	0.93
Oil content	49	4.39	5.66	4.96	0.28

The variation in the chemical composition for reference samples of lupin seeds, as reported in Table 1, 2, 3 and 4, corresponds with results obtained by Glencross *et al*. (2015) for DM (89.20 - 95.00%), ash (1.90 - 6.60%), crude protein (27.70 - 61.30%), crude fibre (17.50 - 43.40%) and oil content (5.00 - 17.10%) and Viljoen (2003) for DM (90.01 - 96.89%), ash (2.60 - 4.43%), crude protein (21.06 - 40.57%) and oil content (3.06 - 11.43%).

The calibration accuracy was determined by the coefficient of determination of the calibration (r^2_{cal}) and the external validation (r^2_{val}). External validation statistics were calculated for the calibration equations developed for DM, ash, crude protein, crude fibre and oil content in the milled lupin seeds as well as the whole seeds. Prediction of an external validation set (n = 38) for milled lupin seeds and whole seeds (n = 12) showed significant correlation between reference values and NIR spectroscopy predicted values, based on the SEP, r^2 and the ratio of standard deviation (SD) of reference data to SEP

(RPD). The number of factors used in the PLS calibration models were those suggested by the Unscrambler software (version 10.3) and are reported in Table 5 and 6.

Table 4 Summary of nutrient composition (%) of lupin whole seed used in the validation set, showing the number of samples (n), minimum, maximum, mean and standard deviation (SD)

Chemical component	n	Min (%)	Max (%)	Mean (%)	SD
DM	12	87.08	89.37	88.67	0.67
Ash	12	2.63	3.00	2.78	0.13
Crude protein	12	21.00	31.44	27.91	2.53
Crude fibre	12	14.29	17.48	15.93	0.94
Oil content	12	4.55	5.55	4.94	0.32

The various calibration equations developed for milled and whole lupin seeds were compared using the r^2 in calibration samples, standard error of calibration (SEC), r^2 in validation samples, standard error of performance (SEP) and standard deviation (SD), standard error of laboratory (SEL) and RPD. Results in Table 5 report the calibration and external validation statistics for milled lupin seeds and Table 6 reports the statistics for whole lupin seeds. The coefficient of determination (r^2) and standard error of calibration (SEC) values for the calibration equations of best fit for each constituent measured in lupins and the coefficient of determination (r^2), standard error of prediction (SEP), RPD values and standard error of laboratory (SEL) for the external validation set, are reported in Table 5 and 6 for milled lupin seeds and whole lupin seeds, respectively.

The coefficient of determination in calibration (r^2_{cal}) values were 0.93 for DM, 0.71 for ash, 0.94 for crude protein, 0.76 for crude fibre and 0.89 for oil content and SEC values were 0.22% for DM, 0.15% for ash, 0.61% for crude protein, 0.69% for crude fibre and 0.23% for oil content in milled lupin seeds. The corresponding values for the chemical components in whole lupin seeds were 0.90 and 0.19% for DM, 0.73 and 0.05% for ash, 0.96 and 0.46% for crude protein, 0.82 and 0.40% for crude fibre and 0.78 and 0.13% for oil content. In the validation tests, the r^2 values for milled lupin seeds ranged from 0.61 for ash to 0.85 for oil content and SEP values ranged from 0.18% for ash to 1.12% for crude fibre. The r^2 and SEP values for whole lupin seeds were 0.66 and 0.08% for ash, 0.96 and

0.53% for crude protein, 0.83 and 0.40% for crude fibre and 0.78 and 0.15% for oil content.

The standard error of prediction (SEP) ratio to standard deviation (SD) ratio was calculated to evaluate the performance of the calibrations. Validation in terms of routine NIR spectroscopy analysis, followed by reference method confirmation of the selected best calibrations, was done by using 38 unknown milled lupin seed samples and 12 whole lupin seed samples which were not included in the calibration set. Values of RPD (Williams, 2001) were calculated to verify the applicability of the calibrations. The RPD is the ratio of the standard error of prediction (SEP) to the standard deviation (SD) of the reference data. As recommended by Williams (2001), RPD ratios can be defined as follows: 0.0 - 2.3 is not recommended, 2.4 - 3.0 is suitable for very rough screening, 3.1 - 4.9 suitable for screening, 5.0 - 6.4 for quality control, 6.5 - 8.0 as process control and a value higher than 8.1 is suitable for any application.

According to these criteria, the RPD values obtained in the study for milled lupin seeds for dry matter (1.87), ash (1.60), crude protein (2.52), crude fibre (1.39) and oil content (2.45), could be considered as moderate NIR spectroscopy models, adequate for screening. Calibration models for whole lupin seeds could be considered as good with RPD values for DM (4.06), ash (1.65), crude protein (4.76), crude fibre (2.39), and oil content (2.13), suggesting that NIR spectroscopy models might be used for routine analysis on these parameters.

Windham *et al*. (1989) stated that if the SEP for validation is less than double the SEL for the primary reference method analysis, the final NIR spectroscopy equation can be accepted for use and the SEP for validation can be used as a reliable indication of the accuracy of the final NIR spectroscopy equation. This rule held true for all the lupin calibrations, which leads to the conclusion that the chemical composition determined by means of NIR spectroscopy is similar to that analysed using conventional laboratory techniques. The SEP obtained in the validation of the single-population equations for DM, ash, crude protein, crude fibre and oil content, was within two times the standard error of the laboratory (SEL), which is the limit usually considered to accept NIRS equations for accurate routine use.

It can be concluded that the values for DM, ash, crude protein, crude fibre, and oil content in whole lupin seeds, showed relatively higher r^2, lower SEP and higher RPD than milled lupin seeds. These results correspond with results obtained by Jansen *et al*. (2013)

who tested the NIR spectroscopy calibration equations on blue lupins (*L. angustifolius*), yellow lupins (*L. luteus*) and white lupins (*L. albus*). The best calibration, cross validation and prediction of independent samples were observed for whole seeds of blue lupins (RMSECV = 0.92; r^2 = 0.82) and RPD values of 2.36 was obtained for crude protein.

Table 5 Calibration and external validation statistics in the development of calibration equations for nutrient composition in milled lupin seed samples

Chemical component	PLS factors	Calibration set		Validation set				
		r^2_{cal}	SEC (%)	r^2_{val}	SEP (%)	SD	RPD	SEL
DM	7	0.93	0.22	0.72	0.55	1.03	1.87	1.80
Ash	7	0.71	0.15	0.61	0.18	0.28	1.60	0.35
Crude protein	6	0.94	0.61	0.84	0.75	1.90	2.52	2.63
Crude fibre	6	0.76	0.69	0.55	1.12	1.56	1.39	2.25
Oil content	6	0.89	0.23	0.85	0.28	0.68	2.45	0.63

Calibration performances were evaluated based on the coefficient of determination (r^2), standard error of prediction (SEP), standard error of laboratory (SEL) and ratio of standard error of prediction to standard deviation (RPD).

The accuracy of calibrations for all constituents, except oil content, was higher when whole seeds instead of milled seed were scanned. The coefficients of determination (r^2) were higher and standard errors of prediction (SEP) reduced in calibrations for whole lupin seeds.

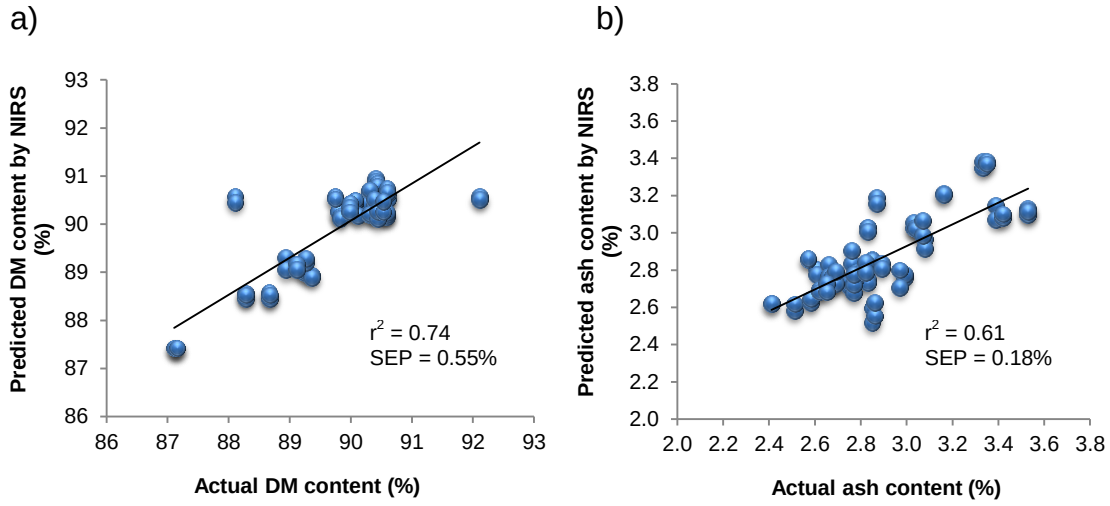
Studies done by Glencross *et al*. (2015) obtained reliable calibration results for lupin kernel meal with respect to coefficient of correlation in validation (r^2_{val}) and standard error of cross validation (SECV) for crude protein (0.91 and 2.00%), crude fibre (0.94 and 0.96%) and oil content (0.78 and 0.48%), respectively. Results obtained by Viljoen (2003) indicated that NIRS can be used for the rapid evaluation of the chemical composition of milled lupin seeds to a degree of accuracy comparable to that of conventional laboratory techniques. Multiple correlation coefficients (*r*) and SEP for the validation sets were as

follows: DM (0.98; 0.28%), ash (0.93; 0.16%), crude protein (0.98; 1.07%) and oil content (0.96; 0.33%).

Table 6 Calibration and external validation statistics in the development of calibration equations for nutrient composition in whole lupin seed samples

Chemical component	PLS factors	Calibration set		Validation set				
		r^2_{cal}	SEC (%)	r^2_{val}	SEP (%)	SD	RPD	SEL
DM	6	0.90	0.19	0.94	0.16	0.67	4.06	0.12
Ash	7	0.73	0.05	0.66	0.08	0.13	1.65	0.08
Crude protein	7	0.96	0.46	0.96	0.53	2.53	4.76	1.03
Crude fibre	7	0.82	0.40	0.83	0.40	0.94	2.39	0.66
Oil content	5	0.78	0.13	0.78	0.15	0.32	2.13	0.28

Figure 2 shows the scatter plots for NIR spectroscopy data versus reference data for (a) dry matter, (b) ash, (c) crude protein, (d) crude fibre and (e) oil content in the external validation set in milled lupin seeds.



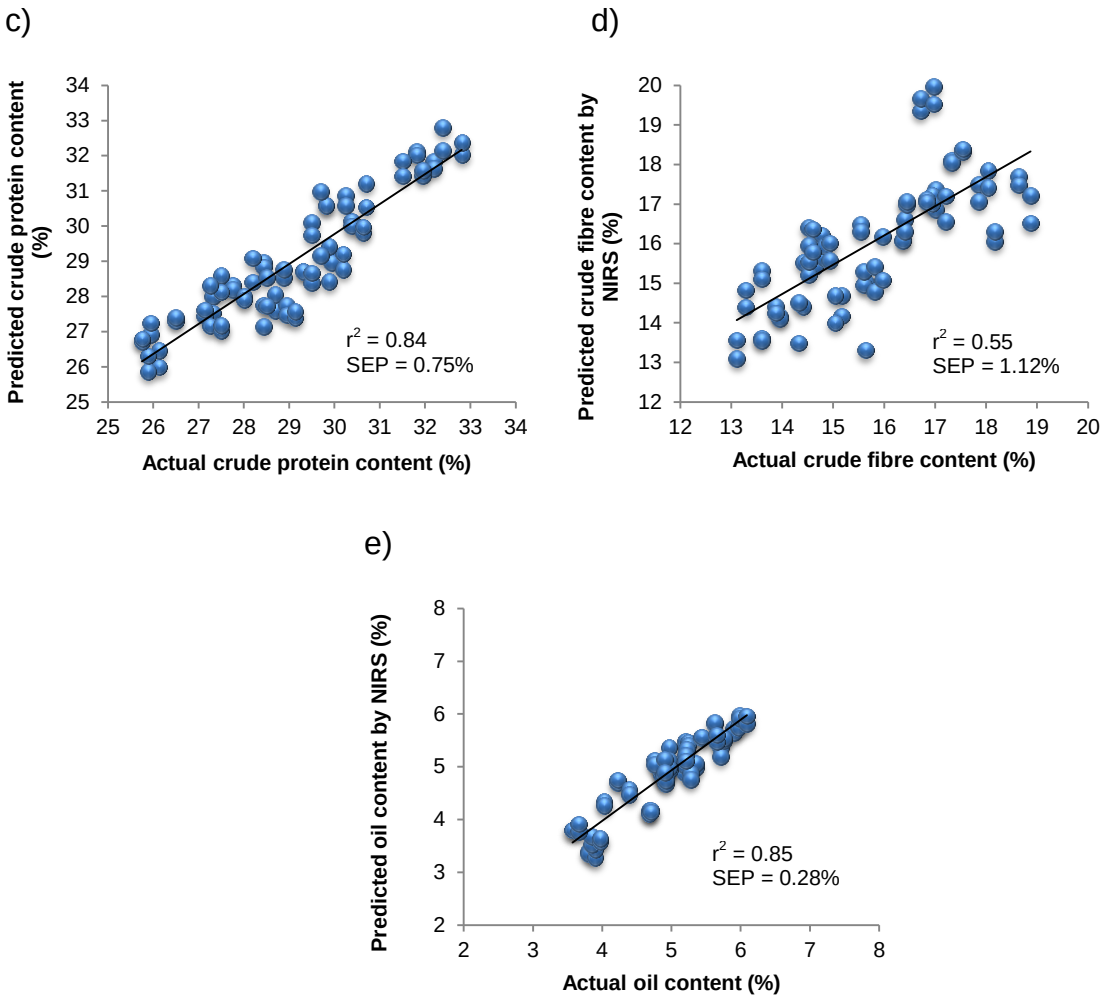


Figure 2 The relationship between laboratory determined and NIR spectroscopy predicted values for (a) dry matter, (b) ash, (c) crude protein (d) crude fibre and (e) oil content in milled lupin seeds

Figure 3 shows the scatter plots of NIR spectroscopy data vs. reference data for (a) dry matter, (b) ash, (c) crude protein, (d) crude fibre and (e) oil content in the external validation set in whole lupin seeds.

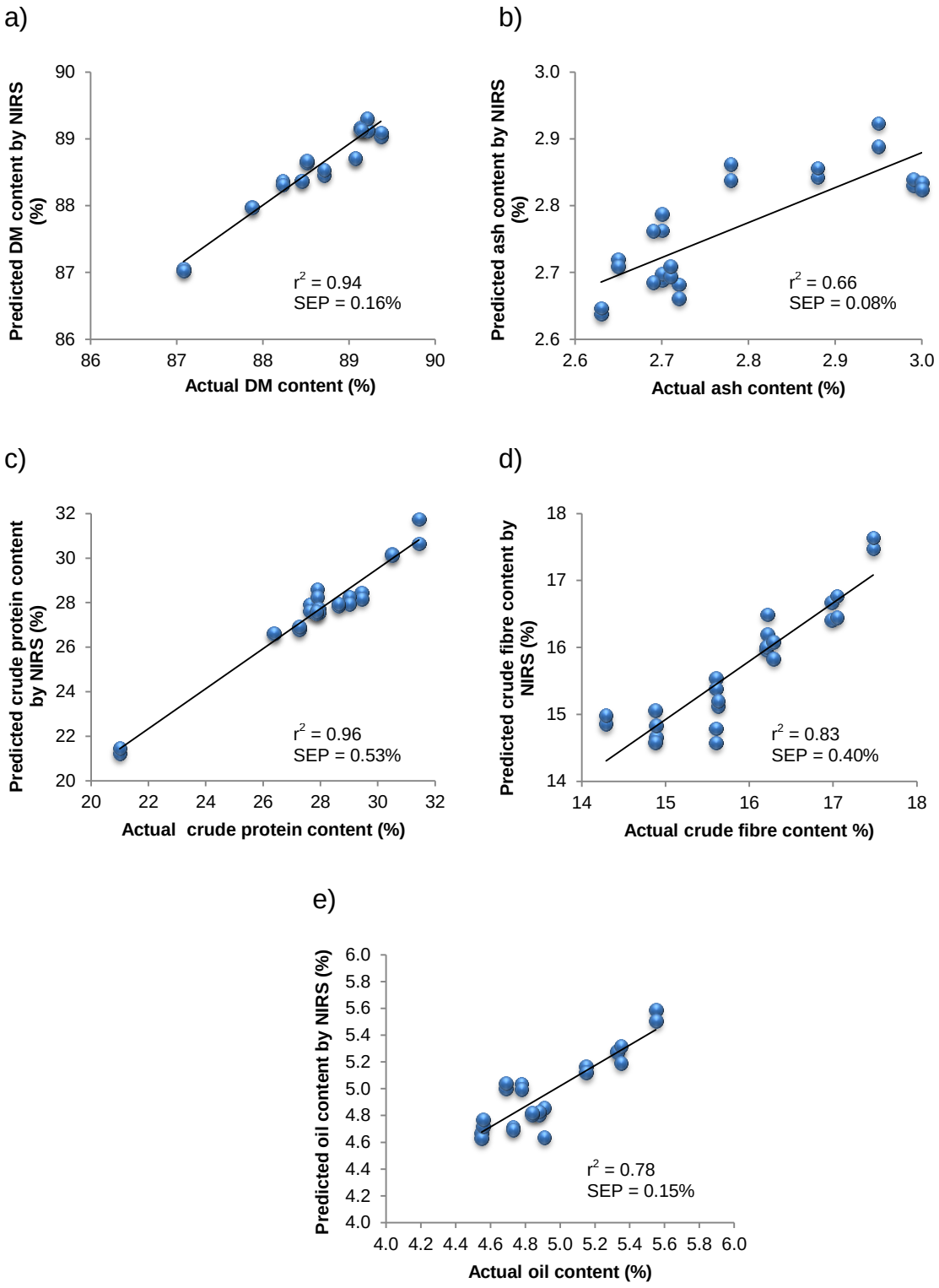


Figure 3 The relationship between laboratory determined and NIR spectroscopy predicted values for (a) dry matter, (b) ash, (c) crude protein (d) crude fibre and (e) oil content in whole lupin seeds

Conclusions

It can be concluded that the NIR spectroscopy technique has a high potential to estimate the accuracy of the chemical composition of whole lupins seed samples compared to milled samples in a non-destructive way and with a high degree of accuracy. The results indicate that there is no need to grind lupin seeds, which will save a considerable amount of time and is less labour intensive. Furthermore, these results have special significance because NIR spectroscopy is a multi-trait technique. Therefore, a simple, rapid and reliable estimation of seed quality traits in this species may be obtained at a low cost, which may have a high impact in applications where large numbers of samples have to be analysed, such as breeding programmes, surveys and quality control.

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CHAPTER 5

PREDICTION OF CHEMICAL COMPOSITION AND AMINO ACID CONTENT OF ALFALFA HAY BY NEAR INFRARED REFLECTANCE (NIR) SPECTROSCOPY

Abstract

The objective of this study was to develop near infrared reflectance (NIR) spectroscopy calibration equations for the prediction of chemical composition and amino acid content from different populations of alfalfa hay (*Medicago sativa* L.), harvested from 6 commercial irrigation farms in the Western Cape and Klein Karoo areas of South Africa. Alfalfa harvested on 10 successive dates over a 10 week period, were evaluated for dry matter (DM), crude protein, ether extract, crude fibre, acid detergent fibre (ADF), neutral detergent fibre (NDF) and essential amino acids (lysine, methionine, threonine and arginine). Samples (n = 60) representing the spectral characteristic of the South African *Medicago sativa L.* hay population were chemically analysed for the development of calibration equations. Samples differed in chemical composition due to collections at different growth stages. Alfalfa hay samples were scanned in reflectance mode on a Perten DA7200 in the near infrared spectral range of 950 - 1650 nm and calibration equations were developed by partial least squares (PLS) regression using cross validation.

NIR spectroscopy calibration equations were developed for the prediction of dry matter (DM), crude protein, ether extract, crude fibre, crude fat, ADF and NDF. Calibration models to predict amino acids included lysine, methionine, threonine and arginine in alfalfa hay were also developed. The current results obtained showed that NIR spectroscopy equations moderately explained the variation in the composition of alfalfa hay. The equations obtained for the prediction of chemical parameters in this study did not explain the major part of the variation existing in the reference data. NIR spectroscopy calibrations could however successfully be used for the prediction of chemical composition if a large enough calibration set is used. The development of accurate calibrations will be of great use in planning feeding strategies for livestock, based on alfalfa hay and forage systems.

Key words: forage quality, alfalfa, NIR spectroscopy, chemical composition, stage of maturity

Introduction

Alfalfa (*Medicago sativa* L.) is a deep-rooted perennial legume pasture plant which is well adapted to a range of climatic conditions and soil types (Devenish *et al*., 2003). Alfalfay hay provides valuable feed for ruminants (González *et al*., 2001) and is an important feed ingredient of monogastric animals, especially ostriches. Feed formulation of the diets of monogastric animals should be based on the provision of essential amino acids and not crude protein, since the nutrient requirements of monogastric animals is based on the amino acids requirement of the body for growth. Lysine is considered as the first limiting amino acid in the nutrition of monogastric animals. Huge variation in crude protein and amino acid content of alfalfa hay, however, exists (Brand *et al.*, 2011). Alfalfa hay is highly digestible (about 65 to 75%) and is a reliable and economic source of crude protein with good levels of metabolisble energy (Devenish *et al*., 2003).

An important factor is that pasture quality changes substantially over a growing season, thus NIR spectroscopy analyses will be a routine requirement if diets are to be of optimal nutritional quality (Corson *et al*., 1999).

Results obtained by Jančík *et al*. (2008) indicate that the protein value of forages is related to the stage of maturity. Maturity of the alfalfa plant leads to a decrease in the crude protein content and to an increase in the concentration of fibre fractions (González *et al*., 2001). Digestibility deteriorates with increased fibre content. However, this does not relate to crude fibre alone, but to the decreased utilization of other nutrients. During the growing season, all plants are subject to changes in composition and consequently in nutritional value and digestibility. Whilst high levels of nitrogen substances are recorded in young plants, these levels drop throughout the plant growth (Písaříková *et al*., 2007) and the amino acid contents decreased with growth stage in alfalfa hay (Homolka *et al*., 2008).

The analysis of chemical composition of feedstuffs is routinely conducted by wet chemistry methods, which are time consuming, costly and in some cases the methods involve hazardous chemicals (Shenk & Westerhaus, 1985). NIR spectroscopy has the benefit of more economical analyses compared to traditional chemical techniques, as there is good potential for substantial economic benefits from balancing the diets (Corson *et al*., 1999). NIR spectroscopy has become widely recognized as a rapid, reliable and non-destructive alternative procedure for the accurate estimation of quality characteristics in feed samples (Batten, 1998) and forages (Shenk & Westerhaus, 1985) that could be applied to the increasing need for effieciency in the feeding of livestock (Shenk &

Westerhaus, 1994). For example, conventional analysis of feed composition (fibre, nitrogen, sugars, lipid and ash) will take about 16 hours, with each of the five assays running concurrently, where this can be completed in 2 - 3 minutes or even a few seconds by NIR spectroscopy on suitably prepared samples (Corson *et al*., 1999). The speed of analysis is the primary advantage of NIR spectroscopy analysis. A finely ground sample of grain or forage can be analysed for multiple nutrients in less than 2 minutes. No special handling of the sample other than grinding is required. The sample does not need to be weighed or corrected for dry matter (Shenk *et al*., 1979).

The NIR spectroscopy technique requires a sample which is exposed to an electro-magnetic scan over a spectral wavelength range of 1100 – 2500 nm, which is the near infrared region. Energy in this spectral range is directed onto the sample and reflected energy (R) is measured by the instrument. The diffuse reflection carries information which identifies chemical bonds within the sample, such as C-H, O-H, N-H and S-H. The reflected energy is stored as the reciprocal logarithm (log 1/R) and the spectra are transformed to provide information about the chemical composition of the sample (Baker & Barnes, 1990).

Sixty alfalfa hay samples were collected from 6 commercial irrigation farms in the Western Cape and Klein Karoo region of South Africa and subjected to dry matter, crude protein, ether extract, crude fibre, ADF, NDF and amino acid analysis.

The aim of this study was to test the potential of NIR spectroscopy as a rapid method to estimate the dry matter (DM), crude protein (CP), ether extract (EE), crude fibre (CF), acid detergent fibre (ADF), neutral detergent fibre (NDF) and amino acids such as lysine, methionine, threonine and arginine, in alfalfa hay.

Materials and methods

Sampling and preparation

Alfalfa (*Medicago sativa* L.) from 6 farms was harvested on 10 successive dates, one week apart, representing different stages of growth. The 60 alfalfa hay samples were collected during 2010 and 2011 from 6 commercial irrigation farms in the Western Cape (Stellenbosch) and Klein Karoo (Oudtshoorn) of South Africa. The samples were collected over a 10 week period to include different growth stages such as boot, early heading, and full heading. The samples included different cultivars, soil characteristics (texture, organic matter, nitrogen content, phosphorus content and pH) and farming management practices

to cover a wide range of chemical and spectra variation. The following cultivars were used in the studies – Magna 60, SA Standard, SA Select and WL525. The alfalfa were cut on several cutting dates, one week apart, starting one week after harvesting, to show changes in composition with increasing plant maturity throughout the growing season. Samples were identified as day 7 to day 70. Plant material represents the whole plant cut, thus including leaves and stems.

Analysis of reference samples

The standard varieties of alfalfa hay (*Medicago sativa* L.) were analyzed for their quality characteristics. The chemical properties of alfalfa hay were determined with the following official methods as described by the Association of Official Analytical Chemists (AOAC, 2012). The oven drying procedure producing the minimim chemical changes in the samples involved drying the samples in an air forced oven at 60°C to constant weight for 48 hours and were ground using a Retsch^{TM} ZM200 sample mill (Haan, Germany) with a 1.5 mm screen. Analytical dry matter was determined by drying a 2 g aliquot of each sample at 100 °C for 24 hours (AOAC, 2012) (Method no. 934.01) and ash by incinerating the dry sample at 500°C for 18 hours overnight (AOAC, 2012) (Method no. 942.05) in a Labcon Muffle furnace RM7 (Labcon, Johannesburg, South Africa). Nitrogen was determined by using a LECO TruMac N Nitrogen Determinator, Version 1.3X (LECO Corporation, Michigan, USA) and converted to crude protein using the factor N x 6.25 (AOAC, 2012) (Method no. 990.03). Crude fibre was determined by the method described by Goering & van Soest (1970), making use of FIWE Raw Fiber Extractor, Velp Scientifica (Velp Scientifica, Milano, Italy). ADF and NDF were determined following the procedure of van Soest *et al*. (1991) making use of FIWE Raw Fiber Extractor, Velp Scientifica (Velp Scientifica, Milano, Italy). Total digestible nutrients (TDN) were calculated by the following equation: TDN = 82.38 – (0.7515 x ((NDF% - 3.41)/ 1.1298)) on an as fed basis (Robinson, 1999). All chemical analysis were performed in duplicate and the results expressed on a dry weight basis.

The amino acid content of the reference samples were analysed with the method described by Grace Davison Discovery Sciences (Grace Davison, 2008) by hydrolysis in HCl medium and high performance liquid chromatography (HPLC) determination. An amount of 0.1g was weighed in a hydrolysis tube and 6 ml of a 6 N HCl and 15% Phenol solution was added. The samples were placed under vacuum after nitrogen was added

and the samples in sealed hydrolysis tubes were placed in an oven at 110 °C for 24 hours for protein hydrolysis to be completed. After this time, the samples were left to cool, were filtered with syringe filter hydrophyllic PVDF (0.45 µm, 33 mm), and decanted into 1.5 ml Eppendorf tubes. Amino acids were derivatised with *o*-phthaldialdehyde (OPA) and 3-mercaptopropionic acid in borate buffer (Agilent Technologies, Waldbronn, Germany) and separated by reverse-phase Dionex HPLC (Dionex Corporation, California, USA) on a 3.9 x 150 mm C18 Nova-Pak column (Waters, Ireland) and a 1.1 ml/min flow rate. Amino acids were identified by L-Amino acid standard (2.5 µmol/ml in 0.1N HCl) (Thermo Scientific, Illinois, USA).

Collection of spectra and calibration development

NIR spectroscopy calibration equations were developed on 60 samples randomly selected from the population set, using partial least squares regression (PLS) with cross validation. The alfalfa hay samples were randomly divided into two sets, the first set of 45 samples was used to develop the calibration models and the second set of 15 samples as the validation set, used to test the accuracy of the calibrations. Alfalfa hay samples were scanned using a diode array analyser (Perten DA7200, Perten Instruments, Huddinge, Sweden) (Perten, 2007). Each sample (35 g) was fitted in a 75 mm diameter cup that rotated during NIR spectroscopy scanning. Absorbance readings at 5 nm wavelength increments were collected over a NIR wavelength range of 950 – 1650 nm. Two scans were conducted on each sample.

Selected spectra were matched with the reference data and calibration models for DM, crude protein, ether extract, crude fibre, ADF, NDF and amino acids (lysine, methionine, threonine and arginine) were developed using PLS (partial least squares) regression and cross validation technique by Unscrambler version 10.3 (Camo, Trondheim, Norway) software. Prior to PLS regression, three mathematical treatments were applied to enhance the quality of the PLS calibrations, which included standard normal variate (SNV), standard normal variate and detrending and 2nd derivative Savitzy Golay. The best calibration was selected by means of the highest correlation coefficient (r^2), rank (number of PLS factors) and lowest standard error of calibration (SEC). The final number of PLS factors selected for each calibration was recommended by the Unscrambler software as being the lowest rank that gives the absolute minimum Y-residual variance. Figure 1a shows the raw NIR spectra and Figure 1b illustrates the

Savitzky Golay 2nd derivative spectra of 60 alfalfa hay samples scanned over the spectral range of 950 – 1650 nm.

a) b)

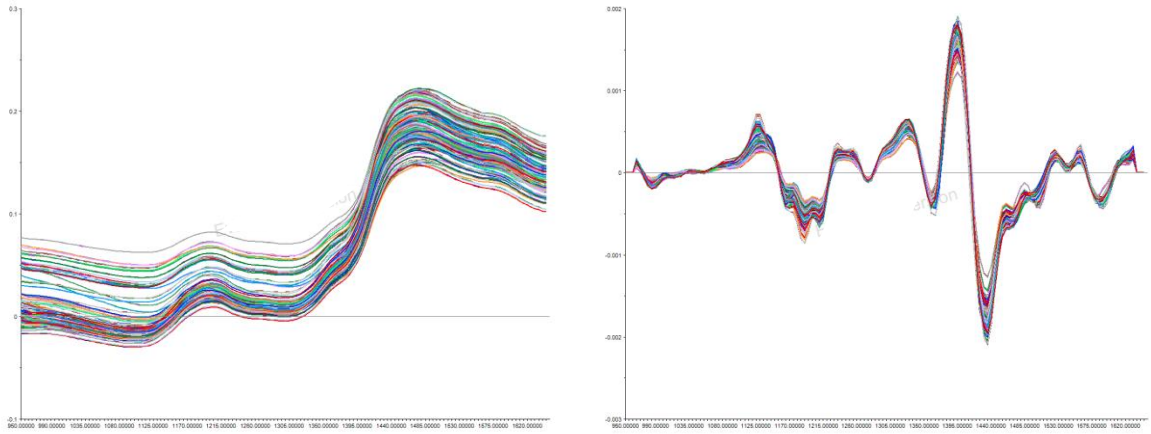

Figure 1 Absorbance spectra for 60 alfalfa hay samples for (a) raw data and (b) Savitzky Golay 2nd derivative spectra in the NIR spectroscopy range

Results and discussion

A wide variation in the chemical composition was expected due to the variety of cultivars, seasons and growth stages used to develop the NIR spectroscopy calibration models. As indicated in Table 1 and demonstrated in Figure 2, contents of crude protein, lysine, methionine, threonine and arginine decreased as the time of sampling proceeded, with dry matter, ether extract, crude fibre, ADF and NDF following the opposite trend. The results in this study were as expected, and in accordance with findings reported by Homolka *et al*. (2008) and Hoffman *et al*. (1993), with the crude protein content that decreased with maturity from 23.0% (day 0), 21.2% (day 6), 16.4% (day 20) to 16.3% (day 30). The crude fibre content increased with stage of maturity with values from 25.5% (day 0), 29.7% (day 6), 40.2% (day 20) to 38.1% (day 30). The percentage contents of the individual amino acids in the alfalfa hay is illustrated in Table 1, which reflected variations caused by different times of harvesting from day 7 to day 70 after cut.

Table 1 Chemical composition of alfalfa hay on air dry basis as influenced by stage of growth (%)

Day of harvest	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56	Day 63	Day 70
DM	90.18	90.36	90.38	90.66	90.57	90.23	90.73	90.85	90.79	91.46
CP	30.25	27.16	25.15	23.58	19.89	19.41	18.29	17.66	19.47	17.44
CF	14.76	17.96	19.16	22.18	24.22	24.16	25.61	28.63	24.59	26.43
EE	2.22	2.21	1.92	2.17	1.99	1.95	2.26	2.55	3.15	3.90
TDN	68.39	66.56	63.95	63.49	61.61	61.46	60.11	58.88	60.26	56.55
ADF	19.78	23.92	27.74	27.68	28.72	28.98	29.88	31.15	28.56	31.08
NDF	24.44	27.19	31.12	31.82	34.63	34.86	36.99	38.75	36.66	42.24
Amino acids										
Lys	1.38	1.22	1.19	1.15	0.95	0.89	0.85	0.78	0.83	0.78
Meth	0.24	0.23	0.23	0.22	0.16	0.16	0.15	0.14	0.15	0.16
Thr	0.93	0.83	0.80	0.78	0.62	0.60	0.54	0.48	0.53	0.46
Arg	0.97	0.84	0.80	0.77	0.60	0.55	0.55	0.54	0.64	0.71

DM - dry matter

CP - crude protein

EE - ether extract

CF - crude fibre

TDN - total digestible nutrients (calculated)

ADF - acid detergent fibre

NDF - neutral detergent fibre

Meth - methionine

Lys - lysine

Thr - threonine

Arg - arginine

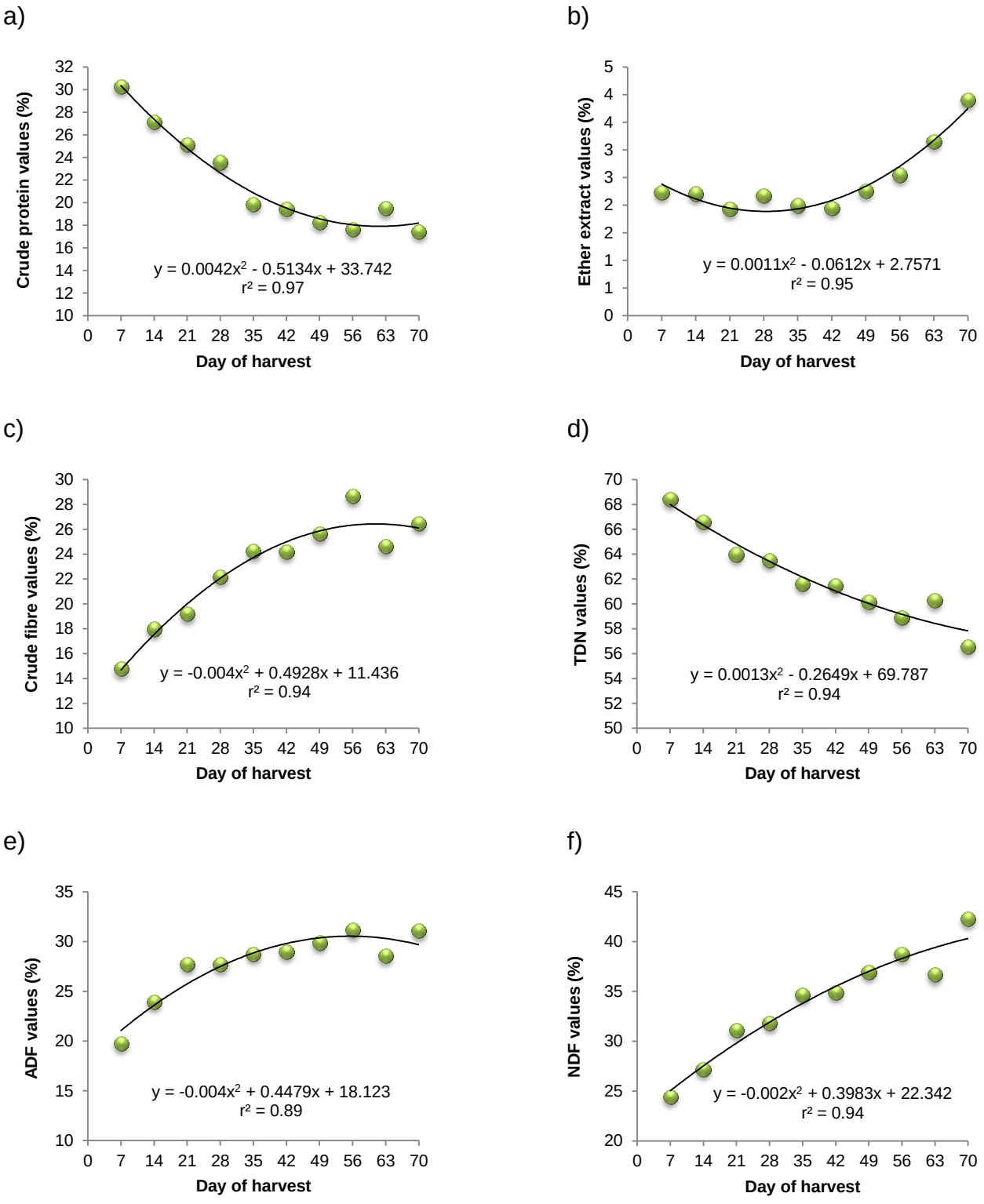

Figure 2 The chemical compositon as influenced by stage of growth (days of harvest) for (a) crude protein, (b) ether extract, (c) crude fibre, (d) total digestible nutrients (TDN), (e) acid detergent fibre (ADF) and (f) neutral detergent fibre (NDF) in alfalfa hay

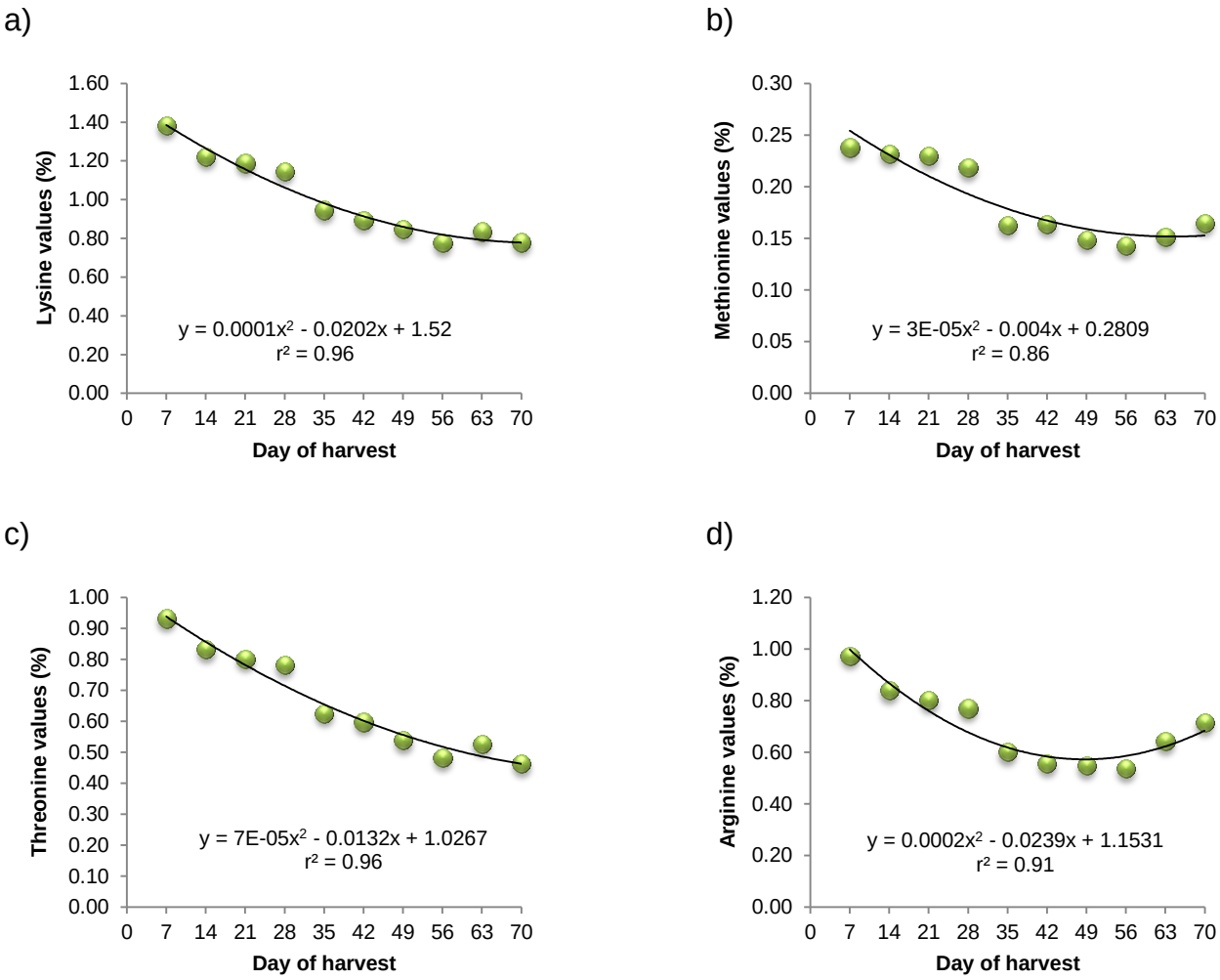

Figure 3 The chemical compositon as influenced by stage of growth (days of harvest) for (a) lysine, (b) methionine, (c) threonine and (d) arginine in alfalfa hay

The nutritive value of lucerne hay as reported in Table 1 corresponds with values obtained by Brogna *et al*. (2009) with results for DM in the range of 87.95 - 96.82%, ash (4.76 - 21.69%), crude protein (5.21 - 26.66%), ether extract (0.60 - 3.73%), ADF (22.93 - 50.85%) and NDF (29.94 - 75.87%). Lucerne meal with 20% crude protein and 40% NDF is ideal for formulating diets for high producing dairy cows (Babinec *et al*., 2001).

Amino acid results obtained by Homolka *et al*. (2008) represented the following ranges: lysine (0.79 - 1.18%), methionine (0.20 - 0.32%), threonine (0.51 - 0.70%) and arginine (0.84 - 1.16%), which corresponds with results obtained in the current study as presented in Table 2. The results reveal a broad range in chemical composition and amino acid contents, especially in the calibration set, which is important for the development of

calibration models. However, the range in the validation set was smaller for chemical composition and amino acid contents, as shown in Table 3.

Table 2 Summary of nutrient composition (%) of alfalfa hay on as fed basis used in the calibration set, showing the number of samples (n), minimum, maximum, mean and standard deviation (SD)

Chemical component	**n**	**Min (%)**	**Max (%)**	**Mean (%)**	**SD**
DM	45	88.67	92.22	90.59	1.06
Crude protein	45	15.75	33.20	22.20	5.14
Ether extract	45	1.49	4.30	2.47	0.71
Crude fibre	45	10.76	40.45	22.47	6.00
TDN	45	50.19	72.40	61.98	4.77
ADF	45	14.57	37.66	27.51	5.45
NDF	45	18.41	51.80	33.45	7.18
Amino acids					
Lysine	45	0.68	1.67	1.02	0.25
Methionine	45	0.04	0.89	0.19	0.24
Threonine	45	0.38	1.10	0.67	0.19
Arginine	45	0.38	1.13	0.70	0.18

DM - dry matter

TDN - total digestible nutrients (calculated)

ADF - acid detergent fibre

NDF - neutral detergent fibre

An independent set of samples were used to validate the NIR spectroscopy calibration models (n = 15). The standard deviation (SD) and ranges of chemical composition in the validation set were for DM 88.79 - 92.11% (SD = 1.10), crude protein 16.22 - 27.87% (SD = 3.60), ether extract 1.60 - 4.56% (SD = 0.82), crude fibre 16.93 - 33.58% (SD = 4.35), ADF 19.14 - 38.57% (SD = 4.95) and NDF 26.55 - 44.52% (SD = 5.06). Corresponding values for amino acids were 0.63 - 1.49% (SD = 0.23) for lysine,

0.03 - 0.77% (SD = 0.21) for methionine, 0.34 - 0.99% (SD = 0.19) for threonine and 0.35 - 0.98% (SD = 0.15) for arginine.

Table 3 Summary of nutrient composition (%) of alfalfa hay on as fed basis used in the validation set, showing the number of samples (n), minimum, maximum, mean and standard deviation (SD)

Chemical component	**n**	**Min (%)**	**Max (%)**	**Mean (%)**	**SD**
DM	15	88.79	92.11	90.73	1.10
Crude protein	15	16.22	27.87	20.71	3.60
Ether extract	15	1.60	4.56	2.48	0.82
Crude fibre	15	16.93	33.58	23.67	4.35
TDN	15	55.04	66.99	61.31	3.36
ADF	15	19.14	38.57	28.45	4.95
NDF	15	26.55	44.52	35.09	5.06
Amino acids					
Lysine	15	0.63	1.49	0.95	0.23
Methionine	15	0.03	0.77	0.16	0.21
Threonine	15	0.34	0.99	0.62	0.19
Arginine	15	0.35	0.98	0.68	0.15

DM - dry matter

TDN - total digestible nutrients (calculated)

ADF - acid detergent fibre

NDF - neutral detergent fibre

Calibration and external validation statistics for NIR spectroscopy models are shown in Table 4, which summarizes the performance parameters, obtained for the calibration equations.

Table 4 Calibration and external validation statistics in the development of calibration equations for nutrient composition in alfalfa hay samples

Chemical component	PLS factors	Calibration set		Validation set			
		r^2_{cal}	SEC (%)	r^2_{val}	SEP (%)	SD	RPD
DM	7	0.62	0.63	0.47	0.80	1.05	1.31
Crude protein	7	0.67	2.86	0.10	3.92	3.59	0.92
Ether extract	7	0.47	0.52	0.04	0.85	0.82	0.96
Crude fibre	5	0.66	3.27	0.36	3.60	4.17	1.16
TDN	4	0.82	1.94	0.80	1.48	3.25	2.19
ADF	5	0.56	3.21	0.43	3.03	3.82	1.26
NDF	5	0.73	3.59	0.60	3.67	3.47	0.95
Amino acids							
Lysine	4	0.54	0.16	0.29	0.19	0.23	1.17
Methionine	7	0.67	0.14	0.35	0.19	0.22	1.16
Threonine	7	0.64	0.11	0.22	0.18	0.18	1.04
Arginine	4	0.59	0.10	0.38	0.12	0.15	1.26

DM - dry matter

TDN - total digestible nutrients (calculated)

ADF - acid detergent fibre

NDF - neutral detergent fibre

Calibration statistics for dry matter, crude protein, ether extract, crude fibre, ADF, NDF and amino acids (lysine, methionine, threonine and arginine) included the calculation of standard error of calibration (SEC), the coefficient of determination in calibration (r^2_{cal}), the standard error of prediction (SEP), and the coefficient of determination in cross validation (r^2_{val}). Calibration performance was assessed by SEC (standard error of calibration), r^2 (coefficient of determination) and SEP (standard error of prediction).

Values for coefficient of determination in calibration (r^2_{cal}) and SEC used as estimates of calibration accuracy for chemical composition and amino acid contents, were as follows:

DM (r^2 = 0.62; SEC = 0.63), crude protein (r^2 = 0.67; SEC = 2.86), ether extract (r^2 = 0.47; SEC = 0.52); crude fibre (r^2 = 0.66; SEC = 3.27), ADF (r^2 = 0.56; SEC = 3.21) and NDF (r^2 = 0.73; SEC = 3.59). The corresponding values for amino acids were as follows: lysine (r^2 = 0.54; SEC = 0.16), methionine (r^2 = 0.67; SEC = 0.14), threonine (r^2 = 0.64; SEC = 0.11) and arginine (r^2 = 0.59; SEC = 0.10).

Williams (2001) suggested the use of RPD or SD/SEP, which is calculated by dividing the standard deviation of the reference values used in the validation (SD) by the standard error of prediction (SEP). A value of RPD higher than 3.0 is defined as satisfactory for the screening in a breeding program and values of 5-10 are defined as adequate for quality control. Williams (2007) stated that the r^2 and the ratio of prediction to deviation (RPD) are the most meaningful statistics for appraisal of analytical efficiency by NIR spectroscopy. Other calibration evaluation statistics used in the present study included SEC and SEP.

The ratio of prediction of deviation (RPD), calculated as standard deviation divided by standard error of cross validation (SECV), was used to evaluate the performance of the calibrations (Williams, 2001). According to these criteria the RPD values obtained in the present study for DM content (1.31), crude protein (0.92), ether extract (0.96), crude fibre (1.16), ADF (1.26), NDF (0.95), lysine (1.17), methionine (1.16), threonine (1.04) and arginine (1.26), showed poor calibration models and might only be used for very rough screening. The lower RPD values can be attributed to a narrow range of the reference values (small SD), or to a large error in the prediction (SEP) compared with the variability of the reference values (Williams, 2001). The models developed in the study on alfalfa hay had relatively low RPD values (less than 3). The poorer calibration performance could possibly be explained by the lack of a large enough sample pool in these parameters in alfalfa hay. These results corresponds with results obtained by Scholtz *et al*. (2009) who obtained calibration equations for DM (RPD = 4.84), crude protein (RPD = 4.57), ether extract (1.82), ADF (3.97) and NDF (3.99).

Figure 3 shows the scatter plots of reference samples measured and predicted using NIR spectroscopy with PLS models in the validation sets for dry matter, crude protein, ether extract, crude fibre, ADF and NDF.

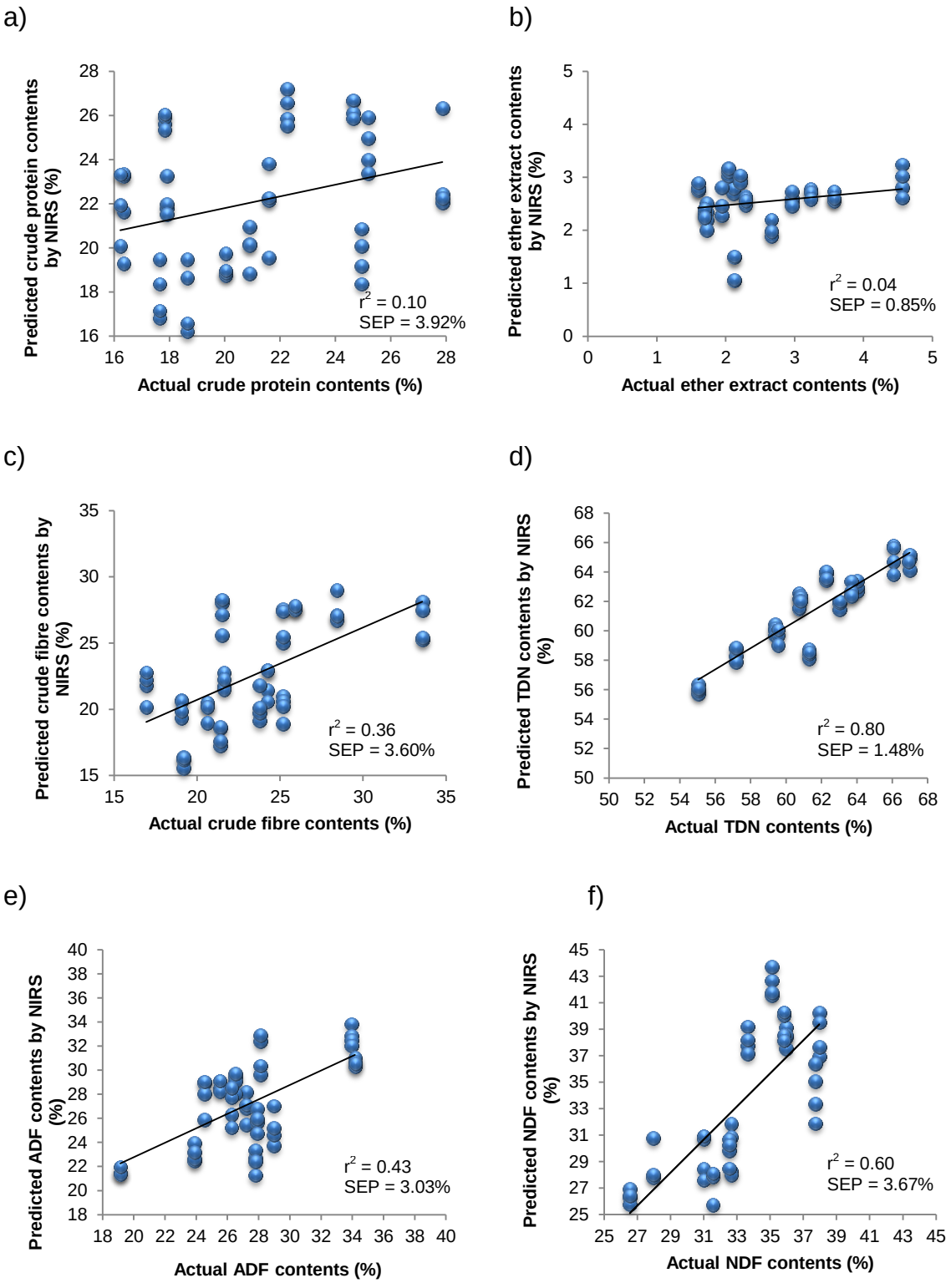

Figure 4 The relationship between laboratory determined and NIR spectroscopy predicted values for (a) crude protein, (b) crude fibre, (c) ether extract, (d) total digestible nutrients (TDN), (e) ADF and (f) NDF in alfalfa hay samples

Figure 4 shows the scatter plots of reference samples measured and predicted using NIR spectroscopy with PLS models in the validation sets for amino acids (lysine, methionine, threonine and arginine).

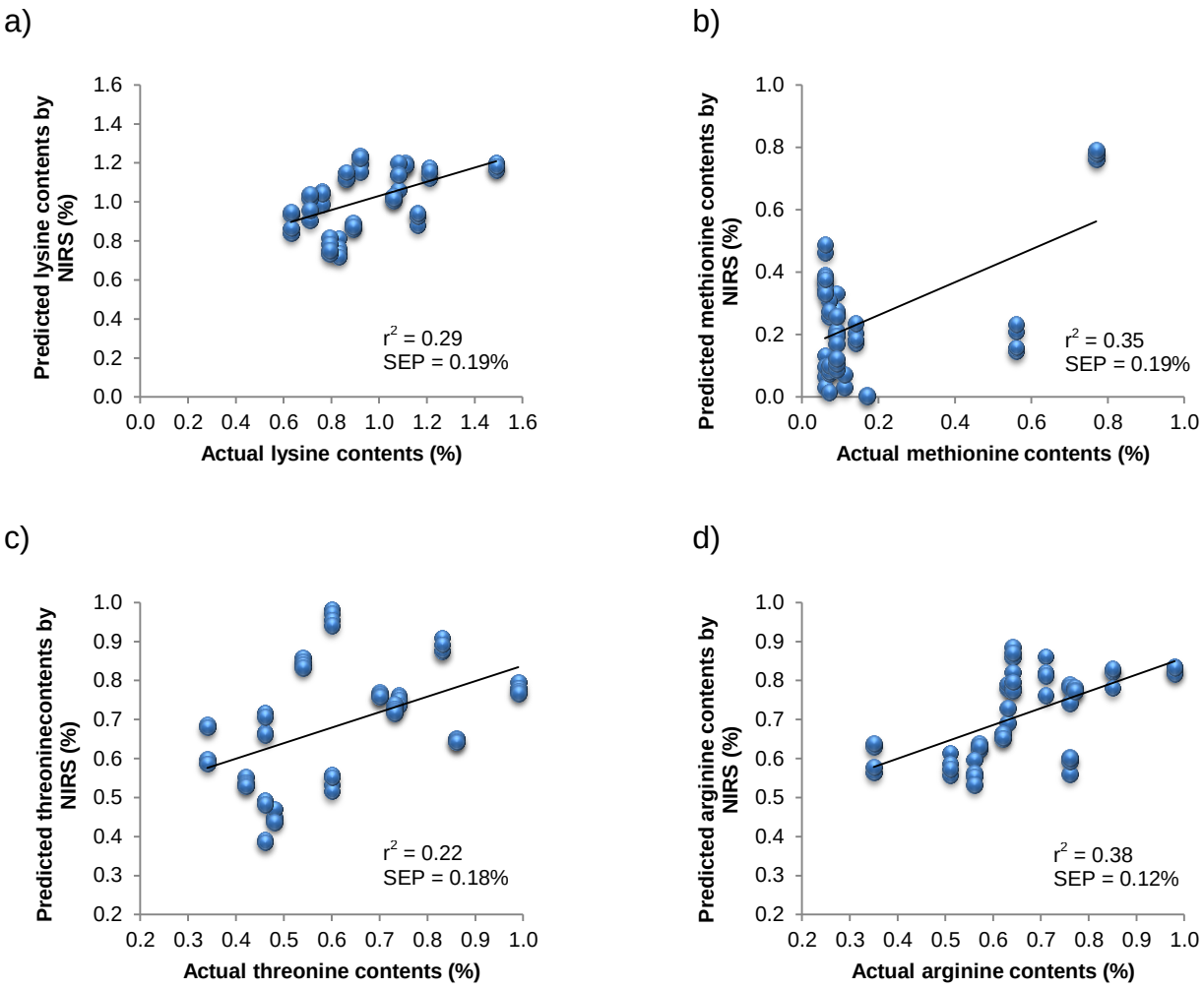

Figure 5 The relationship between laboratory determined and NIR spectroscopy predicted values for (a) lysine, (b) methionine, (c) threonine and (d) arginine in alfalfa hay samples

Conclusions

The results recorded in the present study indicated that the NIR spectroscopy technique moderately predicted the chemical composition (r^2 = 0.04 - 0.80) and amino acids (r^2 = 0.22 - 0.38) on the current set of alfalfa hay samples. However, the usefulness of applying NIR spectroscopy in alfalfa meal has been reported in the literature and more accurate calibration equations can be obtained by adding more samples to the calibration set.

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CHAPTER 6

PREDICTION OF CHEMICAL COMPOSITION AND AMINO ACID CONTENT OF COMPOUND OSTRICH FEEDS BY NEAR INFRARED REFLECTANCE (NIR) SPECTROSCOPY

Abstract

The wet chemical analysis of feed samples is time consuming and expensive. In recent years, near infrared reflectance (NIR) spectroscopy was developed as a rapid technique to predict the chemical composition of feeds and feedstuffs. The prediction and accuracy of NIR spectroscopy relies heavily on obtaining a calibration set that represents the variation in the main population, accurate laboratory analyses, and the application of the best mathematical procedures. In this study, NIR spectroscopy was used to determine the chemical composition of compound ostrich diets. Compound feeds are spectrally complicated due to the wide variety of raw materials that can be used in such feeds, which means an infinite number of combinations are possible. A sample population of 616 ostrich feed samples were available for the development of calibrations and 155 samples were available for the independent validation of dry matter (DM), ash, crude protein (CP), ether extract (EE), crude fibre (CF), acid detergent fibre (ADF), neutral detergent fibre (NDF), calcium (Ca), phosphorus (P), *in vitro* organic matter digestibility (IVOMD), and amino acids such as lysine, methionine, threonine and arginine. The compound ostrich feed samples were analysed with reference analyses and scanned on a Bran & Leubbe InfrAlyzer 500. Coefficient of determination in validation (r^2_{val}) and standard error of prediction (SEP) was satisfactory (r^2_{val} values higher than 0.80) for CP (r^2_{val} = 0.97; SEP = 0.74), CF (r^2_{val} = 0.94; SEP = 1.50%), EE (r^2_{val} = 0.89; SEP = 0.50%), ADF (r^2_{val} = 0.93; SEP = 2.09%), NDF (r^2_{val} = 0.95; SEP = 2.63%) and IVOMD (r^2_{val} = 0.91; SEP = 2.17%), respectively. Less accurate values (r^2_{val} below 0.80) were obtained for DM, ash, Ca and P being 0.57 and 1.04%, 0.67 and 1.30%, 0.43 and 0.59% and 0.49 and 0.11%, respectively. Coefficient of determination in validation (r^2_{val}) and SEP values were r^2_{val} = 0.88; SEP = 0.08% for arginine, r^2_{val} = 0.74; SEP = 0.15% for lysine, r^2_{val} = 0.51; SEP = 0.05% for methionine and r^2_{val} = 0.57; SEP = 0.11% for threonine. In this study the possibility of using NIR spectroscopy to predict the chemical composition of compound ostrich feeds was examined and the results indicate that NIR spectroscopy is a suitable tool for a rapid, non-destructive and reliable prediction of the crude protein, crude fibre,

ether extract, IVOMD, ADF and NDF in compound ostrich feeds. Calibrations can be improved for amino acids if a larger sample pool is used to develop the calibrations.

Keywords: NIRS, ostrich TMR, chemical composition, nutritive value

Introduction

For adequate feeding of livestock, farmers and nutritionists need information about the nutritive value of available feedstuffs (Goedhart, 1990). Livestock selected for high production require an adequate supply of nutrients. This is essential not only for the health of the animals, but also from an economic viewpoint (Givens *et al*., 1997). The cost of feeding is the largest expenditure in ostrich farming. A well-conditioned chick with optimum growth rate and well-nourished, is less likely to succumb to diseases and thus nutrition plays a vital role in ostrich production. Ostriches are monogastric herbivores that must be provided with adequate balanced diets containing optimal nutrients when managed intensively. Nutritional disorders in ostriches, which result in expensive losses to the farmers when it occurs, can be prevented (Aganga *et al*., 2003). The wet chemical analyses of feed samples to determine their chemical composition are time consuming and expensive. Plant breeders, farmers and animal nutritionists require an accurate, rapid, and cost-effective method of assessing the nutritive value of pastures and feeds (Smith & Flinn, 1991). Near infrared reflectance (NIR) spectroscopy provides an opportunity to determine the chemical composition of feedstuffs. Apart from its rapidity, NIR spectroscopy is a physically non-destructive method, requiring minimal sample preparation, with high accuracy. In contrast to traditional chemical analyses, NIR spectroscopy requires no reagents, producing no waste. It is furthermore a multi-analytical technique as several determinations can be made simultaneously and once the NIR spectrometer instrument is calibrated, it is simple to use and operate (Givens *et al*., 1997). For example, conventional chemical analysis of feeds will take two to three days, while a similar analysis can be completed in 2-3 minutes by NIR spectroscopy (Corson *et al*., 1999). However, calibration sets with insufficient distribution of the samples could lead to inaccurate calibrations (Viljoen *et al*., 2005).

The chemical composition of compound ostrich diets varies considerably due to the wide range of raw materials and by-products available. The prediction of the composition of compound feeds is generally less accurate than calibrations to predict the chemical

composition of raw materials. This is mainly due to the variation in range and quantity of raw materials, which may exhibit different spectral characteristics for a compound feed with apparently the same chemical composition (Givens & Deaville, 1999; de Boever *et al*., 1995) and, thus, considerable variation in reproducibility between collected NIR spectra is obtained (de la Roza-Delgado *et al*., 2006). Aufrère *et al*. (1996) stated that NIR spectroscopy is not widely used for concentrates and compound feeds, as a large number of samples are required for the calibration.

Accurate knowledge of the amino acid content of feedstuffs is crucial for the successful formulation of ostrich diets. A lack of the essential amino acids, such as lysine, methionine, arginine and threonine, in the diet, limits the nutritional value of the feed, thereby limiting the growth of the ostriches. Methionine and lysine, for instance, are essential amino acids usually included in poultry diets, since these amino acids are limiting in most feed ingredients (Aganga *et al*., 2003). Yet, amino acid analysis is complicated and labour intensive, requiring 3 days of processing time by using a high pressure liquid chromatography (HPLC). This procedure is costly and time-consuming in comparison to using NIR spectroscopy predictions which are fast and cost effective (de Boever *et al*., 1995). HPLC can be highly variable, due to small sample sizes, multiple steps required for analysis and the hydrolysis procedure which can be very destructive to amino acids, especially methionine and cysteine (Pazdernik *et al*., 1997).

The NIR spectroscopy method relies on the measurement of light absorption by a feed sample when scanned using wavelengths in the near-infrared region (1100 - 2500 nm) with reflectances measured (as log 1/reflectance) at 2nm intervals to obtain the NIR spectra. The resulting absorption spectrum depends on the chemical bonds within the components of the scanned sample and it is therefore possible to identify specific regions of the spectrum correlated with constituents such as starch, fibre or crude protein (Mould, 2003). Calibrations were developed by means of partial least-squares (PLS) regression. PLS regression is the appropriate multivariate calibration technique to avoid the problem of the very high intercorrelation between absorbances (Goedhart, 1990).

The constitution of feedstuffs consists mostly of organic matter. The molecular bonds which are most frequent in feedstuffs are thus bonds between hydrogen, carbon, oxygen, sulfur, phosphorus, and nitrogen. The frequency of the vibration between these molecules is such that these bonds generally absorb light in the near infrared region or the region which extends just beyond red in the rainbow which are visible to humans. NIR

spectroscopy uses the principle that molecular bonds absorb specific frequencies of light to obtain information about the number and type of organic bonds present in a feedstuff (van Kempen, 1996).

Results obtained by de la Roza-Delgado *et al*. (2006) confirmed that NIR spectroscopy could accurately predict the dry matter, crude protein, crude fat, ADF and starch in dairy cow total mixed rations.

A sample population of 771 complete ostrich feed samples was available to develop NIR spectroscopy equations and for use in the independent validation for dry matter (DM), ash, crude protein (CP), ether extract (EE), crude fibre (CF), acid detergent fibre (ADF), neutral detergent fibre (NDF), gross energy (GE), calcium (Ca), phosphorus (P), *in vitro* organic matter digestibility (IVOMD), and amino acids such as arginine, lysine, methionine and threonine. Calibration equations were developed by using a Bran & Luebbe InfrAlyzer 500 NIRS and the samples were scanned between 1100 and 2500 nm by using partial least square regression (PLS) with cross validation.

The aim of this study was therefore to determine if NIR spectroscopy can be used for the estimation of chemical composition and amino acids in mixed and processed ostrich feed samples, which will be a rapid and cost effective method for users who need to rapidly analyse a large number of samples.

Materials and methods

Sampling and preparation

For this study, a very large sample pool was used to develop calibrations from a wide variety of samples, as the samples contain a wide variation of raw materials. A total of 771 samples were collected from separate ostrich feeding trials that were conducted on Kromme Rhee and Oudtshoorn Research Farms in the Western Cape and Southern Cape regions of South Africa. Samples represented feed samples from different growth stages of ostriches, which included pre-starter, starter, grower and finisher phases. The main ingredients used to formulate the diets were: maize meal, soybean oilcake, fishmeal, full fat soya, full fat lupin meal, alfalfa meal, wheat bran, molasses plant oil, monocalcium phosphate, limestone, salt, synthetic lysine, vitamin and mineral premix; these were used in the total mixed ration. Samples were ground using a Retsch^{TM} ZM200 sample mill (Haan, Germany) with a 1.5 mm screen to create consistent particle size meal.

Analysis of reference samples

The compound ostrich feed samples selected for this study varied widely in their chemical composition, as samples of feeds used in different growth stages, such as pre-starter, starter, grower and finisher diets, were collected. A total of 771 compound ostrich feed samples were subjected for chemical analysis for DM, ash, CP, EE, CF, ADF, NDF, *in vitro* organic matter digestibility (IVOMD), Ca, P and amino acids, including arginine, lysine, methionine and threonine.

The DM percentage of the ostrich feed samples was determined by loss of weight after drying a 2 g aliquot of each sample for 24 hours at 100 °C (AOAC, 2012) (Method no: 934.01). The ash content was determined by combustion at 500 °C overnight (18 hours) in a Labcon Muffle furnace RM7 (Labcon, Johannesburg, South Africa) (AOAC, 2012) (Method no: 942.05). Nitrogen was analysed by using a LECO FP428 Nitrogen analyser (LECO Corporation, St. Joseph, Michigan, USA) according to the Dumas Combustion Method (AOAC, 2012) (Method no: 990.03). A factor of 6.25 was used to estimate the CP content. Ether extract was determined by a Tecator Soxtec system HT 1043 (Tecator, Höganas, Sweden), using diethyl-ether as an extraction fluid (AOAC, 2012) (Method no: 2003.06). Crude fibre, ADF and NDF were determined by using a FIWE Raw Fiber Extractor, Velp Scientifica (Velp Scientifica, Milano, Italy). Crude fibre was determined using the method described by Goering & Van Soest (1970), while ADF and NDF were determined according to the method described by van Soest *et al*. (1991).

Ca en P content was determined by the official method of the Agri Laboratory Association of South Africa (AgriLASA), using the dry ashing method 6.1.1 (Palic, 2007) on 0.5 g finely ground ostrich feed samples. The samples were incinerated at 460 °C overnight, and after cooling, 5 ml of 6 M hydrochloric acid (HCl) was added. The sample was placed in an oven for 30 minutes at 50 °C. Subsequently, 35 ml distilled water was added and the solution was filtered into an amber bottle. Ca and P concentrations were measured on an Thermo Electron iCAP 6000 Series Inductively Coupled Plasma (ICP) Spectrophotometer (Thermo Electron Corporation, Milan, Italy), fitted with a vertical quartz torch and Cetac ASX-520 autosampler. Concentrations were calculated by using Merck Titrisol standards with concentration of 1000 ppm (Merck, Darmstadt, Germany) and calculated using iTEVA Analyst software.

The *in vitro* organic matter digestibility (IVOMD) was determined by an adaptation of the method of the two-stage rumen fluid-pepsin technique described by Tilley & Terry

(1963). It involves firstly a 48 hour fermentation by rumen micro-organisms in a buffer solution, followed by a 48 hour pepsin-hydrochloric acid digestion. The residue represents the indigestible part of the sample.

To determine the amino acid content of the reference samples, the finely ground feed samples were analysed with the method described by Grace Davison Discovery Sciences (Grace Davison, 2008), by hydrolysis in HCl medium and HPLC determination. An amount of 0.1 g was weighed in a hydrolysis tube and 6 ml of a 6 N HCl and 15% Phenol solution was added. The samples were placed under vacuum after nitrogen was added and the samples in sealed hydrolysis tubes were placed in an oven at 110 °C for 24 hours for protein hydrolysis to be completed. After this time, the samples were left to cool, were filtered with syringe filter hydrophyllic PVDF (0.45 µm, 33 mm), and decanted into 1.5 ml Eppendorf tubes. Amino acids were derivatised with *o*-phthaldialdehyde and 3-mercaptopropionic acid in borate buffer (Agilent Technologies, Waldbronn, Germany) and separated by reverse-phase Dionex HPLC (Dionex Corporation, California, USA) on a 3.9 x 150 mm C18 Nova-Pak column (Waters, Ireland) and a 1.1 ml/min flow rate. Amino acids were identified by L-Amino acid standard (2.5 µmol/ml in 0.1N HCl) (Thermo Scientific, Illinois, USA). All chemical analysis were analysed in duplicate and expressed on an as is basis.

Collection of spectra and calibration development

The sample population used in the calibration consisted of 616 compound ostrich feed samples while 155 samples were used in the external validation to test the accuracy of the calibrations. The samples were individually presented in closed cups (approximately 6 g) and the scans were acquired using a Bran & Luebbe InfrAlyzer 500 near infrared reflectance spectrometer (IA-500). The samples were scanned in the reflectance mode between 1100 - 2500 nm of the near-infrared region with 2 nm intervals, acquiring 701 datapoints for each sample. Bran & Leubbe SESAME Version 2.00 software (Bran & Luebbe GmbH, Norderstedt, Germany) was used to perform the spectroscopic measurements.

The optimum number of terms in the PLS calibration models were determined by full cross validation as defined by SESAME Version 2.00 software. The 2nd derivative was used as a mathematical treatment in order to correct for baseline effects and to separate overlapping peaks when calibration models were developed (Hruschka, 2001).

Calibrations were developed for the following chemical components: DM, ash, CP, EE, CF, ADF, NDF, Ca, P, IVOMD, and for the following amino acids: arginine, lysine, methionine and threonine. To test the robustness of the calibration method, external validation was performed with a set of samples that did not belong to the calibration set. The reference or laboratory values were determined of the validation set and are samples which did not belong to the calibration set. The aim of the validation set is to test how each of the calibration equations would predict the different parameters and to compare the results obtained with the reference values (González-Martín *et al*., 2006b). The calibration equations were independently validated by using 155 TMR samples and outliers were removed, as suggested by the SESAME 2.0 software. Figure 1 shows the raw NIR spectra of 771 compound ostrich feed samples over the spectral range of 1100 - 2500 nm.

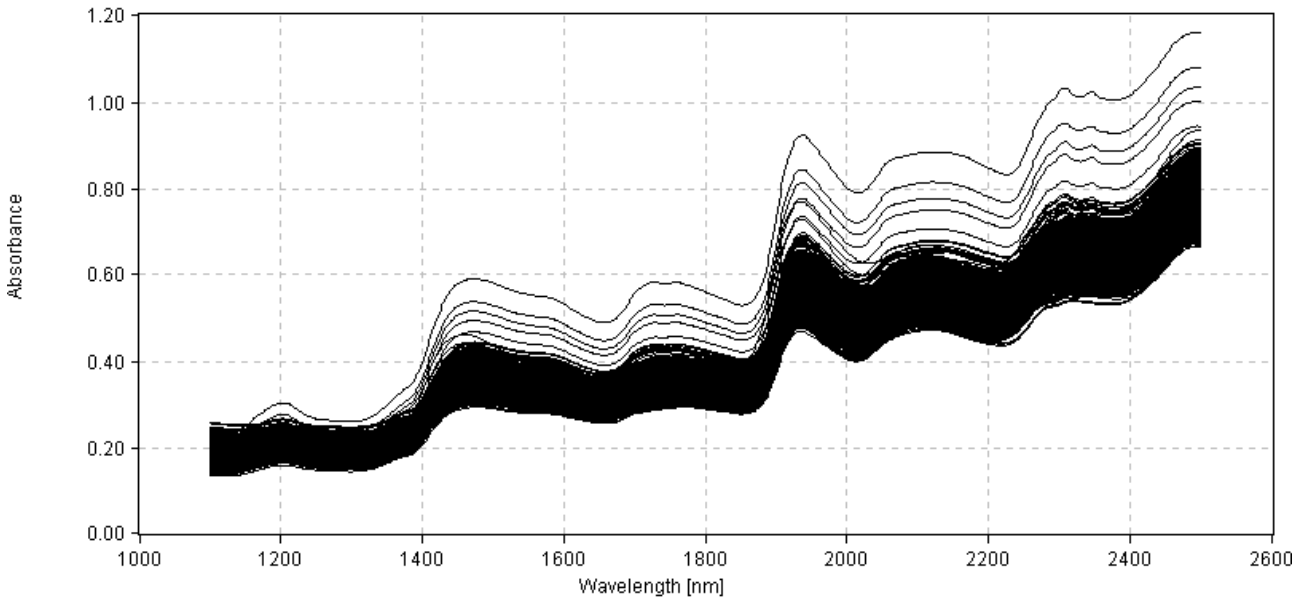

Figure 1 Original absorbance spectra for ostrich total mixed rations scanned between 1100 - 2500 nm wavelengths

Results and discussion

Table 1 indicates the mean values, range and standard deviation (SD) of the nutritive value measured by the reference methods in compound ostrich feed samples used in the calibration set, and shows a wide range in chemical composition, while Table 2 indicates the nutritive values of the samples used in the validation set. Similar feed composition

results for total mixed rations were reported by Aganga *et al*. (2003) and Kritzinger *et al*. (2010).

Table 1 Summary of the nutritive value of ostrich total mixed ration samples as measured by reference methods used in the calibration development, showing the number of samples (n), minimum, maximum, mean and standard deviation (SD)

Chemical component	**n**	**Min (%)**	**Max (%)**	**Mean (%)**	**SD**
DM	384	85.67	94.51	91.27	1.29
Ash	377	4.86	15.59	9.59	2.18
Crude protein	379	5.88	28.93	14.09	3.94
Crude fibre	378	2.91	36.44	17.87	5.96
Crude fat	378	0.47	9.00	2.70	1.45
ADF	616	3.53	46.12	20.38	7.78
NDF	616	7.00	62.72	32.83	11.37
Calcium	380	0.40	5.08	1.97	0.82
Phosphorus	378	0.21	1.38	0.68	0.16
IVOMD	158	60.53	88.97	79.15	6.15
Amino acids					
Lysine	96	0.35	1.48	0.84	0.21
Methionine	96	0.03	0.28	0.09	0.04
Threonine	96	0.15	0.85	0.50	0.13
Arginine	96	0.40	1.14	0.70	0.16

DM - dry matter

ADF - acid detergent fibre

NDF - neutral detergent fibre

IVOMD – *in vitro* organic matter digestibility

Results obtained by Aganga *et al*. (2003) revealed the following ranges in chemical compositions in ostrich diets: dry matter (88.00%), crude protein (12.00 – 20.00%), crude fibre (13.50 - 17.50%), crude fat (2.50%), Ca (0.90 - 3.50%), P (0.50 - 0.65%), arginine (0.46 - 1.20%), methionine (0.19 - 3.50%), lysine (0.60 - 1.00%) and threonine (0.41 - 0.73%). Results in Table 1 correspond with results obtained by Kritzinger *et al*. (2010) with ADF (10.65 - 26.1%) and NDF (21.0 - 43.3%).

Statistics calculated for the calibration included coefficient of determination in calibration (r^2_{cal}) and standard error of calibration (SEC). The prediction accuracy of the models was tested on the validation set using the coefficient of determination in validation (r^2_{val}) and standard error of prediction (SEP). The r^2, SEC and SEP values indicate how well the equations will perform within the same populations (Cozzolino & Moron, 2004). The residual predictive deviation (RPD), defined as the ratio between the standard deviation of the population (SD) and the SEP for the NIR spectroscopy predictions, is a useful statistic that is often applied to evaluate how well a calibration model can predict chemical data (Williams, 2001). If the SEC is large compared to the standard deviations, a relatively small RPD value results and the NIR calibration model is considered not robust (Williams, 2001). The higher the value of the RPD, the greater the probability of the model to accurately predict the chemical composition of samples outside the calibration set. It was reported by Williams (2001) that an RPD value greater than three and less than five is considered fair and recommended for screening purposes. An RPD value greater than five is considered good for quality control. Guidelines for interpretation of *r*, according to Williams (2001), state that a value of 0.83 to 0.90 for r^2 is usable in most applications, including quality assurance. A value of more than 0.98 is usable in any application while r^2 values of 0.66 to 0.81 can only be used for screening and possibly some other approximate applications.

The calibration statistics for the prediction of chemical components in compound ostrich feeds by NIR spectroscopy are presented in Table 3. According to above specifications, the coefficient of determination in validation (r^2_{val}) and standard error of prediction (SEP) was satisfactory. i.e. the r^2_{val} was higher than 0.83 for CP (0.97; 0.74%), CF (0.94; 1.50%), EE (0.91; 0.44%), ADF (0.93; 2.09%), NDF (0.95; 2.63%) and IVOMD (0.91; 2.17%), respectively. The r^2_{val} and SEP values was less accurate (r^2_{val} below 0.80) for DM (0.57; 1.04%), ash (0.67; 1.30%), Ca (0.43; 0.59%) and P (0.49; 0.11%), respectively. Coefficient of determination in validation (r^2_{val}) and SEP values were r^2_{val} =

0.88; SEP = 0.08% for arginine, r^2_{val} = 0.74; SEP = 0.15% for lysine, r^2_{val} = 0.51; SEP = 0.05% for methionine and r^2_{val} = 0.57; SEP = 0.11% for threonine, which indicates that calibrations for amino acids in ostrich total mixed rations is not accurate enough to be used for screening purposes.

Table 2 Summary of the nutritive value of ostrich total mixed ration samples as measured by reference methods used in the external validation set, showing the number of samples (n), minimum, maximum, mean and standard deviation (SD)

Chemical component	n	Min (%)	Max (%)	Mean (%)	SD
DM	94	84.68	97.79	91.24	1.57
Ash	94	4.99	15.86	9.43	2.24
Crude protein	94	5.36	25.24	14.08	4.24
Crude fibre	94	5.09	29.13	17.52	5.65
Ether extract	94	1.04	8.81	2.76	1.48
ADF	155	4.43	35.31	20.20	7.70
NDF	155	10.92	62.33	33.13	12.07
Calcium	94	0.58	4.24	1.86	0.74
Phosphorus	94	0.38	1.05	0.68	0.15
IVOMD	41	60.56	89.50	78.69	7.21
Amino acids					
Lysine	24	0.34	1.50	0.88	0.30
Methionine	24	0.04	0.28	0.11	0.07
Threonine	24	0.21	0.87	0.53	0.16
Arginine	24	0.41	1.31	0.75	0.23

DM – dry matter

ADF – acid detergent fibre

NDF – neutral detergent fibre

IVOMD – *in vitro* organic matter digestibility

Table 3 Calibration and external validation statistics in the development of calibration equations for nutrient composition in compound ostrich feed samples

Chemical component	PLS factors	Calibration set		Validation set			
		r^2_{cal}	SEC (%)	r^2_{val}	SEP (%)	SD	RPD
DM	8	0.77	0.62	0.57	1.04	1.57	1.51
Ash	10	0.87	0.78	0.67	1.30	2.24	1.72
Crude protein	6	0.96	3.94	0.97	0.74	4.24	5.72
Crude fibre	6	0.95	1.36	0.94	1.50	5.65	3.76
Ether extract	6	0.93	0.38	0.91	0.44	1.46	3.34
ADF	4	0.91	2.30	0.93	2.09	7.77	3.70
NDF	6	0.93	3.01	0.95	2.63	12.07	4.59
Calcium	10	0.77	0.39	0.43	0.59	0.74	1.26
Phophorus	10	0.78	0.08	0.49	0.11	0.15	1.35
IVOMD	8	0.95	1.36	0.91	2.17	7.21	3.32
Amino acids							
Lysine	4	0.60	0.13	0.74	0.15	0.30	1.96
Methionine	5	0.68	0.02	0.51	0.05	0.07	1.43
Threonine	7	0.78	0.06	0.57	0.11	0.16	1.51
Arginine	7	0.87	0.06	0.88	0.08	0.23	2.93

DM – dry matter

ADF – acid detergent fibre

NDF – neutral detergent fibre

IVOMD – *in vitro* organic matter digestibility

According to the criteria proposed by Williams (2001), the RPD obtained in the present study for CP content (5.72), crude fibre (3.76), ether extract (3.34), ADF (3.70), NDF (4.59) and IVOMD (3.32), could be considered as good and suggested that NIR spectroscopy calibration models can be used for routine analysis of these parameters. Moderate to poor NIR spectroscopy calibration models, adequate for rough screening, were found for DM (1.51), ash (1.72), Ca (1.26), P (1.35) and amino acids, which ranged from 1.43 - 2.93. The poor calibration performance for ash, Ca and P, could possibly be

explained due to the fact that minerals do not absorb in the near infrared region, which corresponds with results reported by de Boever *et al*. (1995) for compound feeds for cattle.

The coefficient of correlation, *r*, indicates the closeness of fit between the NIRS reflectance and reference data over the range of composition. A high *r* value with a low SEP and bias, together with a slope close to 1.0, means that the NIR spectroscopy results are accurate over the anticipated range and likely to remain so, provided that these statistics were based on a sufficient number of observations (Williams, 2001).

Results obtained by several studies indicate acceptable accurate calibrations in compound feeds for different species for CP, EE and CF, such as cattle, swine, sheep, poultry and rabbits (González-Martín *et al*., 2006b), compound feed for rabbits (Xiccato *et al*., 2003) and cattle, lamb, poultry, pig, ostrich, horse, rabbit, cat and dog feeds (Pérez-Marín *et al*., 2004). Aufrère *et al*. (1996) reported accurate NIR spectroscopy calibrations of energy value in compound feeds for swine and ruminants. González-Martín *et al*. (2006a) obtained comparable results of amino acids with a fibre-optic probe to HPLC analysis in animal feeds such as cattle, swine, poultry and sheep compound feeds.

The relationship between laboratory determined and NIR spectroscopy predicted values for CP, CF, EE, IVOMD, ADF and NDF, is presented in Figure 2 and amino acids (lysine, methionine, threonine and arginine) is presented in Figure 3. Results of NIRS calibration indicate good correlations for CP, CF, EE, IVOMD, ADF and NDF with r^2 values higher than 0.81. The high r^2 values for CP, CF, EE, IVOMD, ADF and NDF indicate very good predictive capability compared to DM, ash, Ca, P and amino acids. Bruno-Soares *et al*. (1998) also reported accurate predictions of CP and CF by NIR spectroscopy, confirming the findings of the present study. NIR spectroscopy is most successful when equations are used on sample sets other than those used in calibration development, so it is necessary to determine whether it is appropriate to analyse a new population with existing NIR spectroscopy equations (Smith & Flinn, 1991).

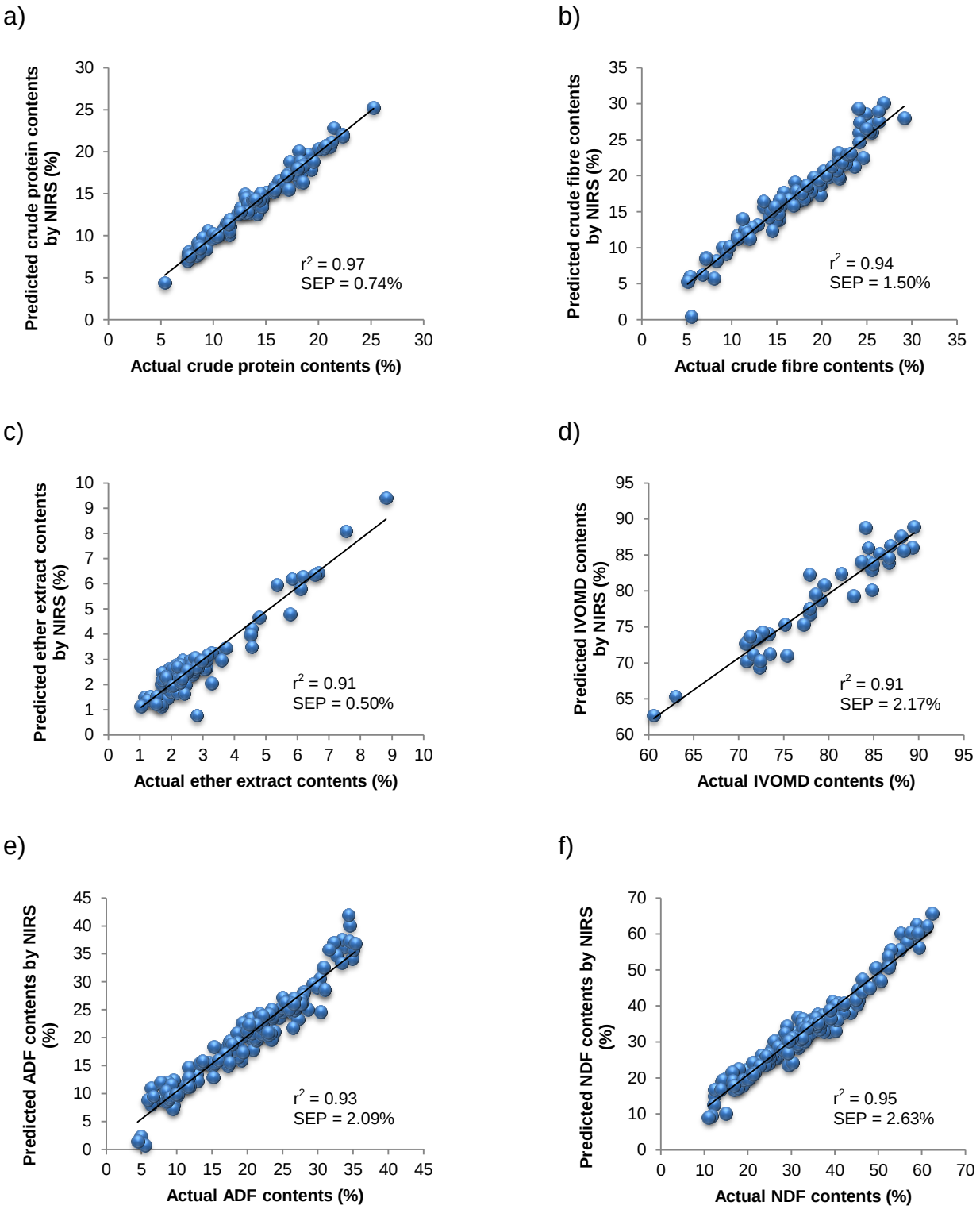

Figure 2 The relationship between laboratory determined and NIR spectroscopy predicted values for (a) crude protein, (b) crude fibre, (c) ether extract, (d) IVOMD, (e) ADF and (f) NDF in compound ostrich feeds

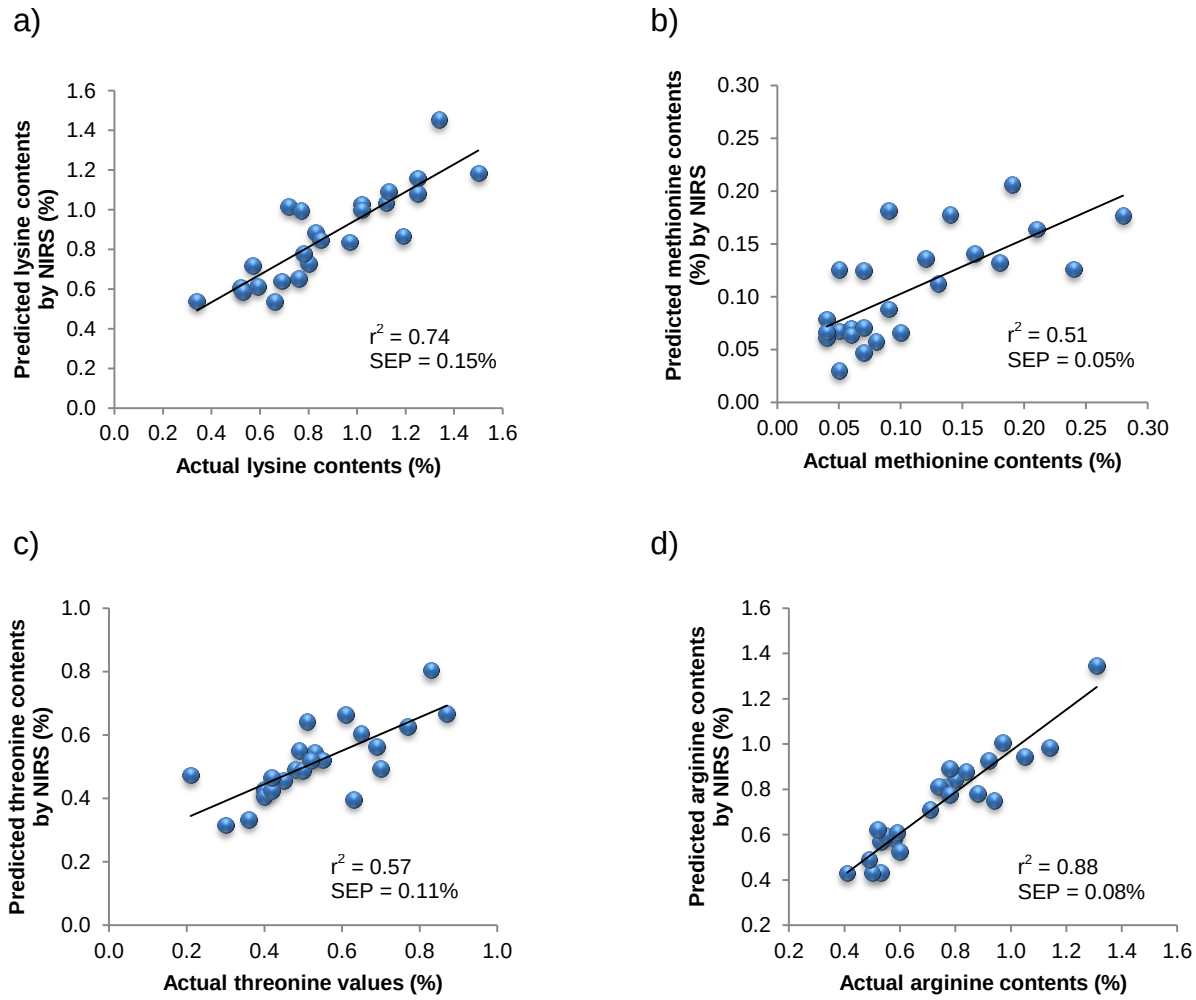

Figure 3 The relationship between laboratory determined and NIR spectroscopy predicted values for (a) lysine, (b) methionine, (c) threonine and (d) arginine in compound ostrich feeds

Conclusions

The above results indicate that good predictions can be obtained from the predictions for CP, CF, EE, IVOMD, ADF and NDF for compound ostrich feeds. Less accurate predictions for DM, ash, Ca, P and amino acids were achieved for compound ostrich feeds. Better calibrations can be obtained for the latter components if separate calibrations are developed for diets with a higher variation in the amino acid contents. The study, however, does indicate that NIR spectroscopy is an accurate technique for the prediction of the most important chemical components in compound ostrich feeds.

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CHAPTER 7

General conclusion and future perspective

NIR spectroscopy has transformed the analysis and nutritional characterization of forages, feeds and feedstuffs as it allows for the rapid and accurate evaluation thereof. The use of NIR spectroscopy in feed analysis has gained recognition due to the fact that its level of accuracy has reached the levels required by the feed industry (Landau *et al*., 2006). The major advantages of NIR spectroscopy is that it provides a more rapid and more cost effective technique and no or minimal sample preparation is required. Several constituents can also be analysed simultaneously (Ruiz, 2001). Making use of a NIR instrument for routine analytical analysis will result in a substantial decrease in traditional wet chemistry analysis (Ruiz, 2001), avoiding both the need for reagents and the production of chemical residues (Garrido-Varo *et al*., 2002).

Disadvantages of NIR spectroscopy entail development of calibrations for each substitute and is time consuming, resource intensive and expensive (Mark, 2012). The accuracy of predictions is dependent upon instrument calibration, supported by good quality assurance methods. The interpretation of data for animal feeding is best achieved using diet balancing software. NIR spectroscopy can be used as an aid to plant selection programmes for improved nutritive value and in the analysis of compound feeds for monogastric animals and ruminants (Corson, 1999). The NIR spectroscopy models need to be frequently updated to accommodate changes in the sample matrix, even for the same type of sample and chemical component. Robust models may require hundreds or even thousands of samples analysed by the traditional chemical methods.

External validation of the procedure should demonstrate the performance of the chosen model using an independent validation set consisting of samples that were not used in the creation of the spectral reference library (Anonymous, 2012).

The results of this study showed that NIR spectroscopy can play an important role in screening of chemical composition and fatty acid analysis in sunflower seed meal, but calibrations can be improved if more samples are added to the calibration model.

The RPD values below 3 in this calibration model indicate that NIR spectroscopy can be used as a rough screening method for milled canola seeds compared to whole seeds.

Calibration models can be improved by using more samples from different cultivars and regions to develop the calibration models.

NIR spectroscopy has high potential to estimate the chemical composition of whole lupin seeds compared to milled seeds, which can discard the need to grind the samples which allows for a more rapid and less laborious method.

According to the results of the study done to predict the chemical composition and amino acid content in alfalfa hay, further development is needed to improve the efficiency of the NIR spectroscopy method. A stable calibration needs a large number of sample analyses in order to cover the wide variability of chemical composition and amino acid content in alfalfa hay. It has important practical implications in that parameters cannot be treated as a constant, but has to be established for each growth stage in further studies.

The results indicate that good predictions can be obtained from the calibration models for CP, CF, EE, IVOMD, ADF and NDF for compound ostrich feeds. Less accurate predictions for DM, ash, Ca, P and amino acids were achieved for compound ostrich feeds. Recommendations for further work in this area would include improving calibrations for the latter components if separate calibrations are developed for diets formulated with a larger variation in amino acid contents.

The results of this work suggest that the calibration and external validation statistics show the potential of NIR spectroscopy technology as a powerful tool for quality control on oilseeds and compound ostrich feeds. Further work must be carried out in order to increase the calibration set to develop a new equation able to cope with more variability.

Increasing pressure to establish environmentally friendly farming practices, will drive future research to develop rapid and accurate methods of determining nutritive value of feeds and feedstuffs and NIR spectroscopy is a great tool to meet the demands.

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