TOTAL GLUCOSINOLATE PRESERVATION AND NEAR INFRARED PREDICTION IN RAPESEED MEAL

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Jayd Marshal Kittelson

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Jayd Kittelson

The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

SUPERVISORY COMMITTEE:

Clifford Hall
Chair

Sensay Simsek

Peter Bergholz

Darrin Haagenson

Approved:

May 12, 2015
Date

Paul Schwarz
Department Chair

ABSTRACT

Glucosinolates (GLS) and their hydrolysis products are of great food and feed safety concern because they are responsible for both the beneficial and harmful properties of GLS-containing plants. Understanding GLS storage stability and total GLS concentration in *Brassica* oil meals is important to ensuring livestock health. The storage stability of GLS and potential of near infrared spectroscopy (NIRS) for screening the total GLS content of various *Brassica* meals obtained globally and over multiple growing seasons was evaluated. Decreases were observed in meal stored at 4 ^{o}C. GLS storage stability within stored *Brassica* meals was possible for 18 months and possibly longer providing the seed meals are protected from exposure to moisture conditions that promote endogenous myrosinase hydrolysis. NIRS spectra data from 400 to 2500 nm were recorded on various *Brassica* meal samples (186) at 2 nm intervals. A global calibration using the *Brassica* database was developed for both ground and unground meal samples with a modified partial least squares regression analysis of conventional laboratory analysis. The optimum NIR calibrations utilized the first derivative and standard normal variate data preprocessing. The ground NIRS calibration for total GLS resulted in a coefficient of determination (R^2) and standard error of the calibration (SEC) and relative predictive determinant (RPD) of 0.96, 6.05, and 6.32, respectively, while the unground NIR calibration had a R^2, SEC, and RPD of 0.93, 7.65, and 5.88, respectively. Finally, a sample set (20) with known GLS concentration (by HPLC) was split and one subset was analyzed via NIR "as is" and the other subset was analyzed by NIR after drying for 16 hours at 60 ^{o}C in a vacuum oven. The dried *Brassica* meal sample set had a slightly better residual (HPLC - NIR) standard deviation (4.57) and average residual (-0.74), compared to the "as is" moisture sample set standard deviation (5.00) and average residual (-1.26). The use of NIRS as a routine analytical method for total GLS

in *Brassica* meals destined for animal feeds has great potential. In addition, the low cost of the NIR analysis may be attractive for manufacturers of *Brassica* meals.

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DEDICATION

This dissertation is dedicated to my wife, Alissa Kittelson, and my daughters, Mallory and Lilyan. Without my wife's support and my family's encouragement, this dissertation wouldn't be possible. A special thanks also to my mom, who inspired me to complete my doctoral degree.

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LIST OF ABBREVIATIONS

ANN .....Artificial Neural Networks

APCI .....Atmospheric Pressure Chemical Ionization

C.....Carbon

eV.....Electron-volts

FIR .....Far Infared

FT-NIR.....Fourier Transform – Near Infrared

GC.....Gas Chromatography

GLS .....Glucosinolates

H.....Hydrogen

Hz.....Hertz

InGaAs .....Indium Gallium Arsenide

IR.....Infrared

HPLC .....High Performance Liquid Chromatography

MIR .....Mid Infrared

MS.....Mass Spectroscopy

MSC .....Multiplicative Scatter Correction

MLR.....Multiple Linear Regression

N.....Nitrogen

NIPALS.....Non-linear Iterative Partial Least Squares

NIR.....Near Infared

NIRS .....Near Infrared Spectroscopy

O.....Oxygen

OSC.....Orthogonal Signal Correction

PCA.....Principle Component Analysis

PCR.....Principle Component Regression

PLS.....Partial Least Squares

R^2.....Coefficient of Determination

RSM .....Rapeseed Meal

RPD.....Relative Predictive Determinant

RMSEP .....Root Mean Square of Error of Prediction

S .....Sulfur

SEP.....Standard Error of Prediction

UV.....Ultraviolet

GENERAL INTRODUCTION

Glucosinolates (GLS) are sulfur containing secondary plant metabolites prevalent in all of the economically important varieties belonging to the genus *Brassica* (cabbage, rape, turnip, and mustard). Over 120 different GLS have been determined and identified but only approximately 16 of those are associated with crop plants (Chen and Andreasson, 2001; Fahey et al., 2001). The GLS molecule consists of a β-thioglucose unit, a sulfonated oxime unit, and a variable side chain derived from an amino acid (Fahey et al., 2001). The differences among the GLS are mostly attributed to the structural substituent possible at the side chain of the β-thioglucose unit.

In the past few decades, an increasing number of researchers have investigated the potential cancer-prevention, crop-protection, and bio-fumigants activities of GLS (Wittstock and Halkier, 2002; Hecht, 2000). GLS are responsible for the pungent, hot flavor characteristics of select *Brassica* seed crops, which are highly valued in mustard spices (Talalay and Fahey, 2001; Hashem and Saleh, 1999; Rosa et al., 1997). *Brassica napus* oil is desirable for industrial applications, which include oil stock for biodiesel production and industrial lubricants/detergents (Friedt et al., 2007). *Brassica* varieties have also been bred to reduce the content of GLS (i.e. Canola), yielding a more palatable oil for human consumption. The remaining meal after oil production is heavily utilized as a protein alternative to soybean meal in the animal feed industry. However, high GLS levels in *Brassica* meals can cause deleterious animal health effects (e.g. induce iodine deficiency, hypertrophy of the liver, kidney and thyroid) and at higher levels mortality (Rowan et al., 1991; Hill, 1991; Burel et al., 2000). These potential negative consequences have been the main basis for GLS analytic, genetic, and processing animal feed research.

Although seed oil is highly valued, *Brassica* oil extraction operations depend on revenues from the meal to maintain profitable margins. Rapeseed meal (RSM) generated from oil extraction is a popular and cost effective source of protein for animal feeds. In addition, rapeseed species, including *B. napus* (rape), *B. campestris* (turnip rape), and *B. juncea* (leaf mustard) can be grown in temperature zones where soybean and sunflower may not survive. RSM global volume growth has increased from 45.2 million metric tons in 2006 to 61.1 million metric tons in 2013 (USDA, 2013). Canola is a type of rapeseed bred to produce low levels of GLS and its meal is an adequate feed protein source replacement for soybean meal. North Dakota is the leading canola producer in the United States, planting over 1.46 million acres in 2012, with over 2 billion pounds utilized in production (USDA, 2013).

In order to capture the protein value in animal feed diets, rapeseed oil processing techniques have been studied to improve GLS reduction by deactivation of the myrosine enzyme, while not compromising the animals ability to digest the meal's amino acids, mainly lysine (Fenwick et al., 1986; Wallig et al., 2002; Minkowski, 2002; Smithard and Eyre, 1986; Quinsac et al., 1994; Liu et al., 1994; Huang et al., 1995; Jensen et al., 1996; Tripathi et al., 2001; Tyagi, 2002; Das and Singhal, 2005) . Because of varying global manufacturing methods, different plant genetics, and fluctuating GLS concentrations between crops and regions, the ability to accurately and rapidly measure GLS in *Brassica* meals is a viable area of study. Agricultural commodity price volatility motivates animal feed producers to become more flexible with the ingredients being utilized to formulate animal diets, but feed safety must also be closely considered when choosing feed ingredients. Strictly purchasing RSM based on nutritional aspects (protein, moisture, fat, amino acids, etc.) may be detrimental to animal health. Without having an accurate, rapid test to determine the GLS concentration of *Brassica* meals, future

animal feeds may become unknowingly contaminated with high concentrations of GLS. In addition, production techniques to deactivate myrosinase may result in reduced protein bioavailability if not adequately monitored.

Analytical techniques have been developed to quantify GLS levels, but chemical analysis of *Brassica* meals can be expensive and time consuming. Direct analytical measurements of intact GLS are accomplished by gas chromatography (GC) (Underhill and Kirkland, 1971; Heaney and Fenwick, 1980), high pressure liquid chromatography (HPLC) (Minchinton et al., 1982; Bjorkvist and Hase, 1988), and/or colorimetric measurement (Thies, 1982). Near Infrared spectroscopy (NIRS) is a rapid analytical alternative that has many advantages compared to direct measurements. NIRS technology allows for considerable time and cost savings per analysis and requires no hazardous chemicals. In addition, samples can be analyzed in their natural form and retained in their natural state. Most research on NIRS for predicting *Brassica* GLS levels has focused on *B. napus* "double zero" (i.e. Canola) wholeseeds (Biston et al., 1988; Williams and Sobering, 1993; Daun et al., 1994) and India mustard (*Brassica juncea*) wholeseeds (Velasco and Becker, 1998; Font et al., 1999). In contrast, the GLS NIRS research for industry rapeseed and mustard meals are scarce and need further attention to determine if NIRS is a viable analytical technique for measuring total glucosinolates in reduced oil *Brassica* meals. This study will review various sample storage techniques for *Brassica* meals to ensure sample integrity during future testing. It will also analyze NIRS ability to predict total GLS concentration in reduced oil *Brassica* meals.

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HYPOTHESIS AND OBJECTIVES

Hypothesis

A quantitative near infrared calibration can be created to measure total glucosinolates in reduced oil rapeseed meals.

Objectives

1. To investigate the potential rapeseed meal total glucosinolate concentration change under various storage conditions for meals obtained by different meal processing techniques.
2. To develop and validate a quantitative near infrared calibration to measure total glucosinolates in rapeseed meal.
3. To determine if different sample processing techniques can improve the near infrared prediction accuracy of total glucosinolate in rapeseed meal.

LITERATURE REVIEW

Infrared in the Electromagnetic Spectrum

Light radiation is considered electromagnetic energy that can be arranged as an electromagnetic spectrum containing properties such as wavelength, frequency, polarity, and intensity. The spectrum is a diverse collection of radiant energy, from cosmic rays to X-rays to visible light to microwaves, each of which can be considered as a wave or particle traveling at the speed of light (Table 1) (Groleau, 2002).

Table 1. Approximate wavelength, frequencies, and energies for selected regions of the electromagnetic spectrum (Groleau, 2002).

Region	Wavelength (Angstroms)	Wavelength (centimeters)	Frequency (Hz)	Energy (eV)
Radio	$> 10^9$	> 10	$< 3 \times 10^9$	$< 10^{-5}$
Microwave	10^9 - 10^6	10 - 0.01	3×10^9 - 3×10^{12}	10^{-5} - 0.01
Infrared	10^6 - 7000	0.01 - 7×10^{-5}	3×10^{12} - 4.3×10^{14}	0.01 - 2
Visible	7000 - 4000	7×10^{-5} - 4×10^{-5}	4.3×10^{14} - 7.5×10^{14}	3-Feb
Ultraviolet	4000 - 10	4×10^{-5} - 10^{-7}	7.5×10^{14} - 3×10^{17}	3 - 10^3
X-Rays	10 - 0.1	10^{-7} - 10^{-9}	3×10^{17} - 3×10^{19}	10^3 - 10^5
Gamma Rays	< 0.1	$< 10^{-9}$	$> 3 \times 10^{19}$	$> 10^5$

Radiation energy is indirectly proportional to its wavelength, and directly proportional to its frequency. Frequency, ν (nu), is the number of wave cycles that pass through a point in one second (Equation 1). It is measured in Hertz (Hz), where 1 Hz = 1 cycle/sec. Wavelength, λ (lambda), is the length of one complete wave cycle and is often measured in centimeters (cm). Finally, c is the speed of light, 3×10^{10} cm/sec.

$$\nu = \frac{c}{\lambda} \text{ and } \lambda = \frac{c}{\nu} \quad (1)$$

Energy is related to wavelength and frequency by the following equation:

$$E = h\nu = hc/\lambda \quad (2)$$

Where h = Planck's constant, 6.6 x 10^{-34} joules/sec.

The infrared region is located towards the middle of the electromagnetic spectrum and covers the electromagnetic wave frequencies in the range of 7.8x10^{-5} to 7.8x10^{-5} cm (Table 2). Three regions are defined in the infrared (IR) range including the far infrared (3 x 10^{-3} to 3 x 10^{-2} cm), mid infrared (3 x 10^{-4} to 3 x 10^{-3} cm), and near infrared (7.8 x 10^{-5} to 3 x 10^{-4} cm) regions. The far infrared (FIR) region is the least energetic. Molecules (inorganic and organometallic) with heavy atoms may absorb FIR waves, inducing intramolecular vibration. FIR is also able to measure the intermolecular stretching and bending of molecules with lighter atoms and molecules with weak bonds such as Van der Waals (Chalmers and Dent, 2006).

Table 2. The infrared regions of the electromagnetic spectrum (Groleau, 2002).

Wavelength	**λ, cm**	**λ, um**	**λ, cm^{-1}**	**Energy**
Visible				
Near IR	7.8 x 10^{-5} to 3x10^{-4}	10 - 0.01	12820 t0 4000	10-37 Kcal/mole
Mid IR	3x10^{-4} to 3x10^{-3}	7 x 10^{-5} - 4 x 10^{-5}	4000 to 400	1-10 Kcal/mole
FAR IR	3x10^{-3} to 3x10^{-2}	10^{-7} - 10^{-9}	400 to 33	0.1-1 Kcal/mole
Microwave				

Mid infrared (MIR) was the first infrared technology primarily used to detect organic compounds and functional groups of unknown mixtures. The technology has been used in many industries, but has not developed as fast as NIR over the past 20 years because of limitations (Richardson and Reeves, 2005). Specifically, compared to NIR, MIR has lower spectral reproducibility and requires a thin sample presentation due to the high absorptivity of organic materials in that region (Prieto et al., 2009).

In the electromagnetic spectrum, the NIR region is closest to the visible spectrum and is the most energetic IR region. NIR was discovered by Herschel in 1800 while measuring the heat produced by filtering the sun light. These experiments lead him to conclude that temperature increased when filtering light from blue (450 – 475 nm) to red (620 – 750 nm). Herschel noticed that the temperature continued to rise even after positioning the thermometer further from the visible red, theorizing that more energy was present beyond what could be seen with the human eye (Robinson, 2013). Further significant research in the NIR region was not done for nearly 150 years, allowing MIR to gain popularity in analytical chemistry. Researchers noticed that the NIR spectra was broad and overlapped at low intensity bands, between 10 and 100 times more attenuated than sharper MIR fundamental absorptions (Dryden, 2003). NIR is able to measure reflectance, which allows thicker samples with minimum preparation to be analyzed. Second, the NIR spectrum can pass through glass and optical glass fibers, allowing for measurements far from the spectrometer, providing significant advantages over MIR (Choquette et al., 2002).

NIR Absorption Theory

Light absorption by some compounds is possible at certain wavelengths, which ultimately changes the atoms energy (McQuarrie and Simon, 1997; Davies and Grant, 1987). In order for a molecule to absorb a photon from the IR, its molecular vibrational frequency must match the

frequency of the IR. Additionally, the radiation and the molecule must interact in a way that the dipole of the molecule changes in the same direction as the electric field vector created as a result of the radiation (McQuarrie and Simon, 1997). The result of this light-molecule interaction is stretching vibrations that affect bond length in two atom molecules, bending vibrations that alter bond angle in molecules with three or more atoms, and molecular rotations (Davies and Grant, 1987). The energy that is absorbed varies on the bond length and the type of vibration, which results in an IR spectrum (Figure 1). The wavenumber, plotted on the X-axis, is proportional to energy. Absorption of radiant energy is represented by a reduction in the curve. Band intensities can also be expressed as absorbance.

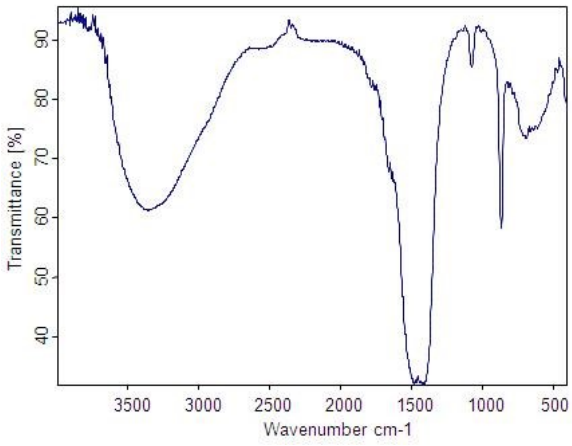

Figure 1. Typical infrared spectrum.

There are two laws which govern the basics of vibrational spectroscopy, Hooke's Law and the Franck-Condon Principle. Hooke's law states that for a simple two body harmonic oscillator, the frequency of vibration (v) is equal to the reciprocal of 2 pi times the speed of light times the square root of the force constant (5×10^5 dynes cm^{-1}) times the sum of the two masses divided by their product. Hooke's Law can be used to calculate the fundamental vibrations for

diatomic molecules in the MIR region, but because NIR is comprised of combination bands and overtones, the Franck-Condon Principle must be understood.

The Franck-Condon principle of anharmonicity introduced relevant concepts to understand the existence of NIR absorption. This principle states that when an electronic transition takes place, the time scale of this transition is so fast compared to nucleus motion that the nucleus is considered static, and the vibrational transition from one vibrational state to another state is more likely to happen if these states have a large overlap. It successfully explains the reason why certain peaks in a spectrum are strong while others are weak (or even not observed) in absorption spectroscopy. The principle accounts for Coulombic repulsion forces between atomic nuclei and kinetic properties of atomic absorption as sources of anharmonicity. In summary, the energy increments are not constant and the updated potential energy function with Franck-Condon principle differs from a harmonic approach.

Anharmonicity also helps to explain NIR overtones, which are the result of bound absorption from ground state to higher non-consecutive energy levels. The NIR spectra contains up to four overtones from the absorptions of the following groups: methyl C-H, aromatic C-H, methylene C-H, methoxy C-H, carbonyl associated C-H, N-H from primary and secondary amides, N-H from amides (primary, secondary, and tertiary), N-H of amine salts, O-H (alcohols and water), S-H and C=O groups (Workman, 2005). It should be noted that absorptions involving hydrogen dominate the NIR spectra because it is a light atom, making it easier to achieve higher vibrational transitions (Davies and Grant, 1987). Combination bands can be found at the highest NIR wavelengths, from 1900 to 2500 nm, and basically involve the same chemical groups as the overtones. They are the result of interactions between molecular vibrational

frequencies, overlapped information from Fermi resonances, and inactive MIR bounds among other phenomena (Bokobza, 1998).

Radiation in Spectroscopy

According to the energy conservation law, when a sample is irradiated with light, part of it is reflected, transmitted and another fraction is absorbed. The proportion of each depends on both the light wavelength/frequency and sample properties (Loudon, 1964). The amount of absorbed energy is explained by the Beer-Lambert Law and is related to sample composition/thickness. Because direct NIR absorbance cannot be measured, reflected and transmitted energies are used. Variability in path lengths, non-homogeneity of samples, and scatter phenomena are a few of the most common factors that limit the direct application of the Beer-Lambert Law.

Diffuse reflectance can be related to the sample absorption but specular reflectance does not provide useful sample information, thus NIR instruments try to minimize this reflectance (Fearn, 2005). After exposure of a sample to NIR, the NIR energy is either scattered or transmitted. A portion of the NIR energy presented to a sample is scatter and the other part is transmitted. Diffuse reflectance relies upon the focused projection of the spectrometer beam into the sample, where it is reflected, scattered and transmitted through the sample material. The back reflected diffusely scattered light (some of which is absorbed by the sample) is then collected by the accessory and directed to the detector optics. Only the part of the beam that is scattered within a sample and returned to the surface is considered to be diffuse reflection. Even with all these sample preparation practices, the raw diffuse reflectance spectra will appear different from its transmission equivalent (stronger than expected absorption from weak IR bands). A Kubelka-Munk conversion can be applied to a diffuse reflectance spectrum to compensate for these

differences (Equation 3). The Kubelka-Munk equation creates a linear relationship for spectral intensity relative to sample concentration.

$$\frac{k}{s} = \frac{(1-R)^2}{2R} = \frac{A}{s} \quad (3)$$

R= Reflectance
k= Absorption coefficient
s= Scattering coefficient
c= Concentration of the absorbing species
A = Absorbance

Physical characteristics affect reflectance measurements especially at higher wavelengths (combination bands region), so any sample change will create an additional source of variability and noise in the measurements (Norris and Williams, 1984). Overall, reflectance measurements show shorter dynamic range compared to transmittance because information provided by diffuse reflectance originates from smaller sample portions and has been attenuated (Corti et al., 1999). Comparison studies in agriculture fields do not lead to a consensus regarding superior performance of any of the two measurement modes (Borjesson et al., 2007).

NIR Instrumentation

For sample presentation, it is common to use open sample cups or sample cells confined by silica or quartz (materials transparent to NIR energy) in laboratory instrumentation for NIR analysis. One of the advantages of NIR energy is its ability to pass through optical glass fibers preserving most of the signal, even if the resulting output intensity is low. The most popular NIR light source is the tungsten halogen lamp (wavelength emission ranges from 320 to 2500 nm). The halogen gas both allows for recycling of the evaporated tungsten and has a longer lifetime compared to traditional tungsten lamps without halogen (Stark and Luchter, 2005).

Prism/gratings diffract the incidental collimated light beam at different degrees, while resolving it into specific wavelengths, are used in dispersive NIR instruments. The dispersion of light can be done either before or after radiating the sample with polychromatic light. Dispersive instruments utilize either monochromators or spectrographs. Monochromators are pre-dispersive instruments that scan a sample with grating mechanical motion. The polychromatic NIR light is able to enter slits and the light rays are made parallel by a mirror. The light contacts the dispersion grating and then hits a focusing mirror. The light is then reflected to a second exit slit to either contact the sample in transmittance mode or hit the single-channel detector in reflectance mode. It is important to note that entrance and exit slits for the monochromator are accurately designed to have precise geometry as they are critical for instrument resolution and effective wavelength bandwidth (Holler et al., 1998). The size of slits affects bandwidth and ultimate application as small slits (around 0.1 mm) give low band width, more dispersion, and high spectral definition and larger slits (around 2 mm) give more intense radiation and are more suitable for quantitative analysis (Holler et al., 1998). In contrast, diode array NIR instruments have a fixed grating and an array of detectors and are post-dispersive instruments that measure all the wavelengths at the same time.

The role of detectors in the NIR equipment is to transform the incident light energy into electric analog signal. This signal is then amplified and transformed into a digital format, which allows for future computer processing. Detectors and amplifiers are considered the most common sources of random noise in NIRS. Instruments are able to reduce this noise by averaging several spectra from the same sample, which ultimately improves the signal-to-noise ratio. Photo-sensitive detector materials are chosen according to the NIR region to be covered. From 400 to 1100 nm, silicon detectors are preferred because they are stable, fast, and sensitive

to low light intensity (Stark and Luchter, 2005). Lead sulfide or indium gallium arsenide (InGaAs) detectors can cover higher wavelength regions compared to silicon. Advanced instruments will include multiple detectors to analyze wider regions. Photodiode arrays spectrographs usually have InGaAs detectors, which offer high signal precision, require minimal signal processing, and have less sensitivity to high light intensities (Greensill and Walsh, 2000).

Fourier Transform NIR

Fourier Transform (FT) has gained recent attention in the NIR industry because the technology offers advantages like high signal to noise ratio, high light outputs due to the absence of slits, fast measurements, and high resolution. Although these attributes are attractive to NIR users, Brimmer et al. (2001) suggests that those advantages are more perceptible when working in the MIR region due to the limitation of higher detector noise relative to the signal when working in the NIR region. Biological materials absorb over broad regions in the NIR, not at discreet wavelengths.

Instead of producing spectrum, the FT-NIR produces an interferogram. NIR interferometers split the NIR light beam in two, where one beam is reflected to a fixed mirror, and the other beam is reflected to a mirror that moves (at controlled speeds). The reflected beams are then recombined in the beam splitter to generate the interferogram signal (Figure 2). When displacing the moving mirror, the path length difference with respect to the fixed mirror changes. This leads to various grades of interference between the two reflected beams, which are correlated with different light frequencies. After the interferogram light reaches the sample, the transmitted or reflected signal is read by the detector in time sequence.

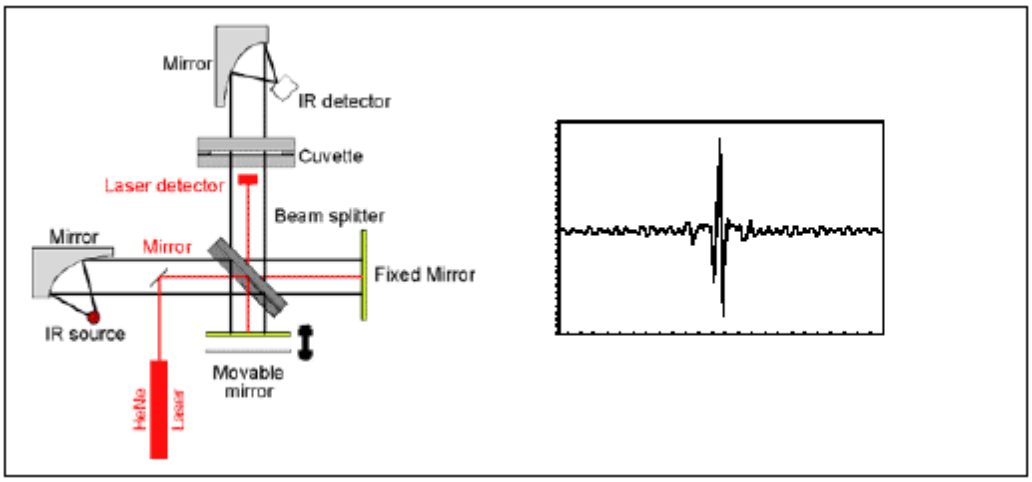

Figure 2. Schematic of a fourier-transform instrument (left) and interferorgram (right) (Hirsch, 2008).

Although interferograms contain information from all the frequencies or wavelengths encoded, it has to be first processed with the fourier transform. The computation takes as an input a time domain wave signal (the interferogram) from which the transform principle states signal is made from an addition of sines and cosines of a set of individual wave frequencies. The processed signal or output looks like the spectra obtained by any traditional spectrometer, but with the expectation of higher throughput and frequency accuracy (Hirsch, 2008). FT-NIR instruments are traditionally more complex and expensive (compared to traditional NIR instruments) and need controlled environments to operate mainly due to their sensitivity to external factors such as temperature.

NIRS Sample Preparation

NIRS is not a stand-alone analytical technique. Its ability to provide rapid analysis depends on the construction of mathematical calibrations. These models are based on statistical relationships developed between a sample's NIR spectra and the constituent value of interest that has been determined by a primary analytical method. The calibrations are then used to predict the

constituents of interest rapidly (within 2 minutes) from the sample spectra generated from the NIR instrument. Accuracy and precision are maintained by periodical, on-going monitoring and updating of the calibrations. Without computing capabilities and multivariate methods, NIRS applications would not be possible.

When defining the samples that will be used in the calibration, it is important that the samples are representative of the population. The single most important step in making any NIR calibration is the selection of training samples (Fearn, 2005). At least 20 and 30 samples should be taken for feasibility studies and initial calibrations, but more robust calibrations may need well over 100 samples (Williams, 2001). The factors that determine the required sample size include the sample set's homogeneity, matrix complexity, particle size and variety. Calibrations of homogeneous mixtures may require smaller calibration sets than agriculture samples of high compositional complexity and heterogeneity, such as whole grains or forages. An ideal calibration set should cover the chemical, spectral, and physical characteristics of the population to be analyzed and avoid future extrapolations when predicting new samples (Fearn, 2005). In addition, steps should be taken to ensure the reference samples are uniform.

It is important to note that NIRS rely primarily on wet chemistry methods to build the calibrations, so the quality of the reference data greatly influences the NIRS calibrations. Understanding the accuracy of the primary method is important for creating realistic expectations for the NIR model. Also, detecting and removing true outliers from the calibration set can improve the NIR model. There are many tools used by chemometricians (model residuals, Leverage) to assist in determining and removing calibration outliers (Haaland and Thomas, 1988).

NIR Spectroscopy Pretreatments

Pretreatments or spectral preprocessing methods are a set of mathematical procedures on spectra before developing a calibration model. These mathematical preprocessing techniques are not required, but are usually utilized by chemometricians to alleviate the variability in sample thicknesses and differences in light scattering. This helps to sustain a more linear relationship between analyte concentration and absorbance by reducing background noise and increasing the signal from the chemical information. Selecting appropriate pretreatements for a given dataset is based on chemometrician experience and trial and error. Although there are several techniques, most of them are variants from the basic well-known pretreatment methods for noise reduction, baseline correction, resolution enhancement, and centering/normalization (Wold et al., 1987).

NIR Noise Reduction Methods

Noise arises from physical and/or chemical processes during the scanning process. The noise can range from low (e.g. instrument drift during scanning) to high (e.g. NIR instrument's detector and electronic circuits) frequencies. An often integrated approach to remove spectra noise is accumulation-average processing. If this reduction is not enough, smoothing techniques (e.g. moving-average method and Savitzky-Golay method) are often implemented to remove high-frequency noise.

Mean averaging is a filtering method that performs smoothing after calculating the average from the data points inside the window and replacing the value of the first data point with the mean (Wold et al., 1987). This process is achieved by moving the window one data point and carrying out the same procedure until the end of the spectra. The wavelet transform technique is able to remove the high and low frequency noise as well as the localized noise due to light scattering. Smoothing methods can only remove low frequency noise. Wavelets operate

by taking the spectrum and transforming it into the wavelet domain and returning it to the spectral domain (Chau et al., 1996; Stork et al., 1998).

Baseline Correction Methods

Baseline correction methods are utilized in NIR model development to eliminate complex matrix interference that is mainly caused by unknown components and/or background in the analysis of derivative spectra. Derivative methods have long been used in NIR spectroscopy as pretreatment methods for resolution enhancement as well as baseline correction (Griffiths, 1987). For calibration development, three mathematical treatments are usually tested: 0,0,1,1 (raw data), 1,4,4,1 (first derivative) and 2,5,5,2 (second derivative), where the first number is the derivative order, the second is the gap between data points for subtraction, and the third and fourth are the numbers of data points used for smoothing. The first derivative removes the displacements from the baseline while the second derivative corrects the terms that vary linearly with the wavelength. The second derivative is most often used in modeling as the superimposed peaks in an original spectrum turn out as clearly separated downward peaks in the second derivative spectrum. Gap-segment and Savitzky-Golay are the best known techniques to perform spectra derivatives pretreatments (Wold et al, 1987).

Another baseline correcting method is Multiplicative Scatter Correction (MSC). This strategy is most often utilized to correct vertical variations in the baseline and inclination of the baseline (Geladi et al., 1985). Light scattering has wavelength dependence different from that of chemically based light absorbance, so data from many wavelengths can be used to distinguish between light absorption and light scattering. The MSC is able to correct spectra according to a simple linear univariate fit to a standard spectrum. The Orthogonal Signal Correction (OSC) procedure has also been introduced to remove the interfering variations present in NIR spectra.

This is achieved by removing a small number of factors that account for the total variation in the spectrum matrix and are orthogonal to the dependent variable to be modeled (Wold et al., 2006).

Resolution Enhancement Methods

In order to unravel overlapping bands and elucidating the existence of obscure bands, resolution enhancement methods (e.g. derivative methods, difference spectra, mean centering, and fourier self-deconvolution) are widely administered (Siesler et al., 2002; Ozaki et al., 2001). Mean centering is an adjustment to a data set to reposition the centroid of the data to the origin of the coordinate system. This is achieved by subtracting the average from all spectra values at each individual data point from each spectrum, moving the mass of the data center to the space coordinates origin without affecting the distance between the points. Centering the data to the mean value can help to reduce the model complexity, which can reduce the number of latent variables. Standard normal variate is an adjustment to a data set that equalizes the magnitude of each sample (Mark, 1991). This is achieved by centering and scaling the spectrum of each sample. The mean of the spectrum is subtracted from each spectrum wavelength, and the result is divided by the standard deviation of the spectrum.

NIR Modeling

Sample absorbance is expected to be linearly related to the compound to be measured according to Lambert's law. Popular linear modeling techniques include multiple linear regression (MLR), principle component regression (PCR), and partial least squares (PLS). Nonlinear modeling techniques are also widely used in the food/feed industry and include local modeling and artificial neural networks (ANN). Principle component analysis (PCA) will be discussed independently as it is usually coupled with other regression analysis.

Principal Component Analysis

PCA is a technique that summarizes the variance-covariance matrix of spectral variables, reducing the dimensionality of the data but keeping the main information from the variables (Hotelling, 1933). Geometrically, PCA changes the initial highly correlated axis of the data to a smaller set of axis, called principal components (PCs). The data is projected on new orthonormal axes which are built as linear combinations of the original variables (i.e. wavelengths). The algorithm finds these new axes seeking for the orthogonal directions which explain the maximum data variability. The first PC will be drawn following the direction which explains the highest variability. The second PC will seek the second direction of maximum variability under the constraint of being orthogonal (perpendicular) to the first PC. The third PC will seek the third direction of maximum variability being perpendicular to the first and second PCs and so on.

Loadings define the new axes, which are the cosines of the angles that each PC forms with the old axis (i.e. wavelengths). After data normalization (by autoscaling or mean centering) the data can be projected to the new PC axes according to Equation 4.

$$\boldsymbol{T = X*P + E} \tag{4}$$

Where X is the original data matrix and P is the matrix of loadings (P). T is the score matrix, or the new values that the original data acquire on the new axes and E is the matrix of residuals. The number of PCs that can be calculated depends on either the number of initial variables or samples, but commonly only up to 20 are calculated from NIR data. The first PCs are the most important and provide the most information as they explain the most sources of variability.

Multiple Linear Regressions

Mutliple Linear Regression (MLR) models are best utilized when the number of measured wavelengths is relatively low or advanced wavelength selection methods are used. MLR is a generalization of the univariate inverse method based on least squares fitting of y to x**.** Each independent variable is correlated with the reference value and its correlation is measured with the coefficient of determination (R^2*)*. This is done in a stepwise manner through creation of a sequence of MLR equations. At each step of the sequence, the variable that makes the greatest reduction in the error sum of squares of the sample is added to the regression equation. This action is duplicated until some stopping criterion is met or all the predictors are processed. This results in all possible linear regressions on all subsets of the available independent variables to be tested. The subset of predictors that produces the lowest standard error is reported. It is important to note that MLR techniques can be apt to over-fitting when a significant amount of irrelevant information or too many predictors are incorporated into the calibration (Davies and Grant, 1987).

Partial Least Squares and Principal Component Regression

Partial least square (PLS) and principle component regression (PCR) models are grouped together because they both utilize wavelength correlation. The PLS supervised regression is more popular than unsupervised PCR models because PLS calculates the principal components considering both the spectral data matrix and the reference values. PCR is considered a direct application of the principal component analysis method. After the spectral data is presented to the new orthogonal non-correlated dimensional axis, multiple linear regression least squares is conducted between the projected data and the reference values. Both methods carry out regression on data projected to a new dimensional space, but the new space coordinates created

in PLS regression take into account the information provided from the reference value matrix resulting in latent variables instead of principal components.

Latent variables are calculated by maximizing the covariance between X and Y. In PLS, the X matrix is transformed to new variables as scores (T), loadings (P), and vectors called weights (W). The scores are orthogonal and estimated as linear combinations of the original variables with the coefficients weights. Weights are calculated from the X matrix directly and used to compute T. PLS is usually preferred over PCR because the algorithm is faster, models have higher precision, and the technique provides more harmonious calibration models with less latent variables (Kalivas and Gemperline, 2006). The main algorithm to perform PLS calibration is non-linear iterative partial least squares (NIPALS).

Local Modeling

Local calibration modeling is a more recently developed calibration strategy compared to PLS. It can be used to develop quantitative calibrations for both linear and nonlinear data systems. This technique deploys multiple PLS algorithms as it uses a subset of the calibration data to make a dynamic model for each unknown sample based on the similarity of the unknown sample to those samples in the calibration database. In contrast to the 'static' calibration models such as PLS, PCR and ANN where the whole calibration data set is used for modeling construction and then used to predict unknown samples, local calibrations create subsets from the calibration library to build small calibrations and predict unknown samples.

Local modeling also offers increased flexibility because users can choose different parameters to optimize the local procedure including the number of samples to select from the calibration library, the minimum number of predicted values generated with the first few PLS terms to be excluded from the calculation of the final predicted values, and the maximum

number of PLS terms to be used in the calibration (Shenk et al., 1997). Furthermore, a local calibration database is usually very robust and composed of a compilation of similar products with expected variation, and therefore suitable for supporting multiple instruments, predicting multiple species and reducing calibration maintenance. However, a local calibration requires more space and a large number of computations for each prediction in routine analysis.

Artificial Neural Networks

Artificial neural networks (ANN) emulate the structure and the operation of a biological nervous system. The construction of a neural network is a training process achieved by learning from the spectra data and corresponding reference values in the calibration library. The architecture of a neural network usually consists of multiple layers including an input layer, a hidden layer, and an output layer. The input layer consists of a few nodes representing the spectra latent variables. The derived latent variables are then used as new eigenvectors of the original spectra and the future inputs of the neural network. The input neuron signs are propagated through the hidden layer to the output neuron and then the error is calculated and back-propagated (in 3-layers) to iteratively adjust weights and biases to minimize the error in the prediction (Demuth et al., 2008). If needed, trans-sigmoid and linear transfer functions can be used in the hidden and output layers to construct non-linear mapping from input to output and improve the model prediction capability (Maier and Dandy, 2000).

The development of the ANN model requires similar division of the calibration and validation set, the data preprocessing methods, and outliers definition as defined for the PLS model. The original data set is divided into three subsets: stop set, validation (testing) set and calibration (training) set. Similar to the use of cross-validation in PLS and PCR modeling, the stop set is used to determine the optimal calibration parameters, such as terms or factors, and

prevent over fitting of the training sample set. The neural network with the minimum prediction error on stop set and validation set are chosen for the final calibration modeling.

NIR Model Validation and Statistics

The NIR calibration was evaluated by calculating the standard error of the calibration (SEC) which is the variation within the reference population not explained by the calibration (Equation 5).

$$\mathrm{SEC} = \sqrt{\frac{\sum (L - M)^2}{n - 1 - p}} \tag{5}$$

Where L is the laboratory reference values, M is the NIR measured value, n is the number of samples and p is the number of terms in the model.

After the NIR model is built, the calibration should be validated with samples not included in the initial calibration to understand the model's effectiveness. If independent validation is not possible, a cross-validation can be utilized to assess the calibrations performance, although any statistics reported cannot be directly compared to a true independent validation. The coefficient of determination (R^2) is a metric used in statistical model analysis to assess how well a model explains and predicts future outcomes. It is indicative of the level of explained variability in the model. It is important to note that if the reference value range is limited the R^2 may not be a good estimation of model performance (Fahey, 2002). The standard error of prediction (SEP) provides information regarding calibration precision (Equation 6). When reporting the SEP, the bias must be reported as the SEP calculation is dependent upon systematic error.

$$\mathrm{SEP} = \sqrt{\frac{\sum (L - M)^2}{n}} \tag{6}$$

Where: L = laboratory reference, M = NIR measure reference, n = number of samples, p = number of terms in the model.

The square root of mean standard error of prediction (RMSEP) is related to SEP and Bias according to Equation 7. Because RMSEP accounts for bias and provides information regarding calibration accuracy, it can be reported alone, especially when the bias is small (Davies and Fearn, 2006).

$$\boldsymbol{RMSEP^2 = SEP^2 + Bias^2} \tag{7}$$

The final statistic to be discussed is the ratio of performance of deviation or relative predictive determinant (RPD), which is dimensionless and applies only to NIR spectroscopy (Equation 8).

$$RPD = \frac{Sd_y}{SEP} \tag{8}$$

Where Sd y = standard deviation of reference values from the validation set and SEP = the models standard error of prediction. The ratio correlates the model's ability to predict future data in relation to the initial variability of the calibration data. A RPD value below 0 and 2.3 indicates that the calibration is not usable. An RPD value between 2.4 and 3.0 reveals the possibility of distinguishing between high and low values, whereas a value between 3.1 and 4.9 makes approximate quantitative predictions possible. For an RPD value between 5.0 and 6.4, the

prediction is classified as good, whereas for an RPD value above 6.5, the prediction is classified as very good and can be used for process control (Williams, 2001).

Brassica NIR Modeling

Because of its commercial importance, most of the *Brassica* research involving NIR technology has been limited to *B. napus* (i.e. double zero/canola) intact seeds (Biston et al., 1988; Michalski and Kryzmanski, 1988; Williams and Sobering, 1993; Daun et al., 1994). Limited studies using NIRS for determining the quality parameters of other *Brassica* species has been reported and has been focused on *B. campestris*, *B. juncea*, and *B. carinata* (Velasco and Becker, 1998; Font et al., 2004) whole seeds. Research regarding GLS concentration and detection from *Brassica* species after production (meals) destined for animal feed consumption and *Brassica* leaves is limited (Fahey, 2002).

Petisco et al. (2010) reported an effective PLS global NIR model (R^2 of 0.99, RPD of 10, and SEP of 2.57 μmol/g) using intact seeds. This model was developed utilizing 189 samples of multiple cultivars of rapeseed intact seeds ranging from 15.8 to 97.9 μmol GLS/g. These results were similar to the statistics achieved by Velasco and Becker (1998) who developed a total GLS model (R^2 of 0.99 and SEP of 4.1 μmol/g) with 270 samples from several *Brassica* species using intact seeds having GLS ranging from 6 to 193 μmol/g. Lower R^2 values (0.90-0.97) and greater SEP values (5.01-5.80 μmol/g) have also been reported (Evans and Bilsborrow, 1989).

Rapeseed

Rapeseed is believed to have originated in the Mediterranean region of south-western Europe through spontaneous hybridizations between turnip rape (*B. rapa*) and cabbage (*B. oleracea*) genotypes (Kimber and McGregor, 1995; Friedt et al., 2007). The species is divided into two subspecies, namely *B. napus ssp. napobrassica* and *B. napus ssp. napus*. The

chromosome number varies for the different species within the *Brassica* genus (Figure 3). The species *B. campestris, B. oleracea* and *B. nigra* are diploid whereas *B. napus, B. carinata* and *B. juncea* have tetraploid sets of chromosomes. Rapeseed cultivars are classified as winter or spring types according to their vernalization requirement in order to induce flowering. Winter cultivars are usually higher yielding than spring cultivars, but they can only be grown profitably in areas where they regularly survive the winter (Butruille et al., 1999). Oilseed rape is cultivated predominantly as winter or semi-winter forms in Europe and Asia, respectively. Spring-sown canola types are more suited to the climatic conditions in Canada, northern Europe and Australia (Friedt et al., 2007). The majority of oilseed rape cultivars are pure lines derived from breeding schemes designed for self-fertilizing crops (Snowdon et al., 2006). In nearly all species, the seeds are mostly spherical with a diameter between 1.2-2.8 mm and a weight of 1.5-7 mg (Wagner et al., 1999) (Table 3).

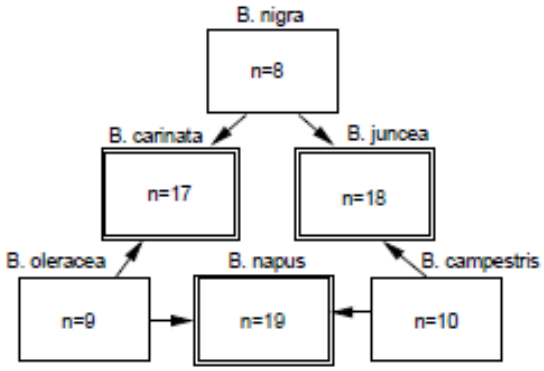

Figure 3. Specific relationship between rape and related species (Holmes, 1980).

Table 3. Diameter, color and thousand grain-weight of rapeseed seeds (Wagner et al., 1999).

Species	Diameter (mm)	Color	Weight (mg)
B. napus	1.8 – 2.8	Black – Brown	3 - 7
B. campestris	1.2 – 2.5	Red – Brown - Black	1.5 – 3.5
B. juncea	1.4 – 1.8	Dark Red – Brown	1.8 – 4.3
S. alba	2 – 2.8	Light Yellow - Yellow	6 – 10.7

Canola was developed from rapeseed by plant breeders to obtain plants with low levels of erucic acid in the oil and low levels of GLS in the non-oil part of the plants (Thomas, 2005; Newkirk, 2009). Rapeseed with low levels of erucic acid (< 2%) and GLS (< 30 μmol/g) are called "double-zero" or "double-low" rapeseeds or 00-rapeseeds in Europe, but in North America, such varieties are called canola (Newkirk, 2009). New varieties of rapeseeds are constantly being developed to improve yield, disease and insect resistance, oil quality, and meal quality (Thomas, 2005; Diederichsen and McVetty, 2011). Recent rapeseed varieties have been modified to improve the nutritional content (i.e. vitamins, minerals, amino acids, etc.) and reduce the fiber and antinutrients of the meals destined for animal feed (Newkirk, 2009).

Rapeseed Processing

Rapeseed ranks second in world-wide oilseed production and protein meals (behind soybeans) and contributes approximately 13% of the total oilseed and protein meal production in the world. Global production of rapeseed exceeds 60 million metric tons with the major producers being Europe, China, Canada, and India (USDA, 2013).

The process of oil extraction from rapeseed seeds includes seed grading, drying, handling, seed cleaning/preparation, extraction, and processing of oil (Salunkhe et al., 1992; Unger, 2011) (Figure 4). Oil extraction can be categorized by two processes: with or without

solvent extraction (Adams et al., 2006). If solvent extraction is not used, oil may be expelled from the seeds using double pressing (Adams et al., 2006; Spragg and Mailer, 2007; Newkirk, 2009).

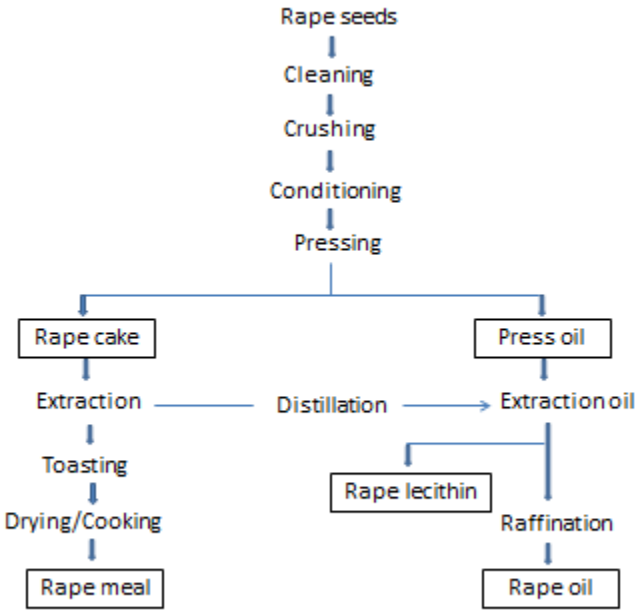

Figure 4. Basic rapeseed processing steps.

Rapeseed is usually received at the extraction production facility, graded and checked for quality. The graded seed is then roughly cleaned (with industrial aspirators, sifters or shakers) to remove foreign material (plant stalks, grain seeds, etc). After cleaning, the seed is heated to about 30-40 ^{o}C to prevent seed shattering. The preheated seed is then flaked on two sets of roller mills, with the second set adjusted to a tighter clearance than the first. After being rolled, the flaked seed is then heated to about 75-100 ºC in cookers. This process helps to rupture the remaining intact cells to release oil, coalesce small oil droplets to larger ones, coagulate protein for better diffusion during solvent extraction, and to adjust the moisture content of the seed prior

to solvent extraction. The cooker is also responsible for deactivating any enzymes that may be present in the flaked seed (mainly myrosinase and lipase) to ensure quality oil and meal.

In order to improve the efficiency of the solvent extraction process, the heat-conditioned seed is sometimes passed into expellers to reduce the oil content of the seed from about 42 % (8 % moisture basis) to 16-20 % (Salunkhe et al., 1992; Newkirk, 2009). The presscake is then conveyed to the solvent extractor. In the extractor, the solids are solvent-washed in stages, first with hexane already high in oil content (miscella) and then with progressively leaner miscella and, finally with pure hexane (Newkirk, 2009). This leaches the oil content in the solid material (i.e. meal) down to less than 3 % (Sauvant et al., 2004; Newkirk, 2009). The meal and the miscella are now "stripped" of the solvent to recover solvent-free meal and oil. The solvent-saturated meal is conveyed to a desolventizer, which is a vertical tank equipped with heated trays and agitators. Reduced pressure and sometimes live steam are used to evaporate the hexane and to dry the meal. The hexane and moisture vapors are condensed, the water and hexane are separated, and the hexane is reused. Several stages of heating and drying are applied to reduce the hexane content to negligible levels and moisture to 8-11 %.

To achieve the best meal quality, the process must be well controlled with respect to temperature (110 ºC max.) and time. Usually, the final treatment in desolventizing is a cooling stage. Cooled meal may be ground to a uniform particle size, or pelleted, ready for storage and marketing. It is important to note that excessive heating in the processing of rapeseed can cause protein damage and digestibility reduction of amino acids in animals (Bell, 1993; Newkirk et al., 2003). In addition, additives such as gum and soap stocks may be added in the process to reduce the dustiness of the meal. This addition may increase the total oil content in RSM by 1 to 2 % (Spragg and Mailer, 2007; Newkirk, 2009).

The oil extraction process has an effect on the total GLS content of the meals because of varying oil extraction conditions (Table 4). Solvent-extracted meals on average contain higher amounts of GLS compared to dehulled extracted meals. While expeller extracted meal contains less GLS than solvent extracted. The extraction condition also affects GLS metabolites present in the meal (Bourdon and Aumaitre, 1990; Glencross et al., 2004).

Table 4. Effects of oil extraction process on glucosinolate (μmol/g) content of meal (Tripathi and Mishra, 2007).

Extraction process	Rapeseed type	Total GLS	ITC (mg/g)	OZT (mg/g)
Solvent extracted	RSM 0	166	3.5 (2.1 - 5.5)	9.2 (5.2 – 14.9)
	RSM 00	38	1.3 (0.3 - 2.1)	2.4 (0.3 – 4.5)
	Canola Meal	3.62	ND	ND
Dehulled extracted	RSM 0	151	4.7	11.5
	RSM 00	30	0.8 (0.2 – 1.5)	1.6 (0.3 – 3.7)
Expeller extracted	RSM 0	36	1.3 (1.3 – 1.4)	3.5 (3.2 – 3.8)
	Canola Meal	1.1	ND	ND
RSM: rapeseed meal, GLS: glucosinolates, ITC: isothiocyanates, OZT: oxazolidinethione, ND: not determined				

The other main category of rapeseed processing is double pressing. This process is similar to the prepress solvent extraction process, but solvent extraction, desolventization, drying, and cooling is not used. Instead, the pre-pressed seeds go through a second press to remove additional oil. The oil concentration in rapeseed expellers from this process is between 8 and 10% (Newkirk, 2009). This virgin oil is in demand by consumers of organic and natural foods, and usually the price of cold pressed oil is greater than that of conventional canola oil (Przybylski and Michael, 2011).

Rapeseed Nutrient Composition

Rapeseed nutrient composition (Table 5) can be influenced by variety, environmental conditions during crop development, harvest conditions, and processing of the seed and meal (Barthet and Daun, 2011; Bell, 1993; Newkirk, 2009). Canola and RSM from yellow-seeded varieties have greater concentration of oil and crude protein, and less crude fiber than meal obtained from black-seeded varieties (Trindade et al., 2012; Slominski et al., 2012). Meal from the solvent extraction procedure have greater concentration of crude protein and amino acids and less concentration of oil than meal that has been expelled (Spragg and Mailer, 2007; Newkirk, 2009).

Table 5. Chemical composition of soybean, canola, and rapeseed meals (as fed) (NRC, 2012).

Soybean meal	Soybean meal	Rapeseed meal	Canola meal
Dry Matter (%)	89.98	88.70	91.33
Digestible Energy (kcal/kg)	3,619	2,771	3,272
Metabolic Energy (kcal/kg)	3,294	2,532	3,013
Net Energy (%)	2,087	1,505	1,890
Crude Protein (%)	47.73	33.70	37.50
Fat (Ether extract %)	1.52	2.30	3.22
NDF (%)	8.21	28.30	22.64
ADF (%)	5.28	19.60	15.42
Crude fiber (%)	3.89	12.40	10.50
Calcium (%)	0.33	0.83	0.69
Total Phosphorous (%)	0.71	1.14	1.08

Rapeseeds typically contain between 40-45 % oil, which is used in multiple applications for both food (i.e. canola cooking oil) and industrial (i.e. biodiesel, industrial lubricants, and detergents) applications (Friedt et al., 2007). Once the oil is extracted, the remaining meal is mostly used in livestock feed mixtures. The inclusion of RSM in animal feed diets can be compromised by the presence of GLS, a group of secondary compounds typical for crucifer plant

species. GLS protect the plant from insect pests but high intakes of seed meal GLS and their degradation products in livestock feeds can cause palatability and health issues, thereby limiting the use of RSM in animal feed. Hence, there is a strong interest in seed-specific optimization of GLS levels and composition, in order to improve the nutritional value of RSM without compensating the disease and pest resistance properties in the crop (Wittstock and Halkier, 2002).

Glucosinolate Structure

More than 120 different individual GLS have been isolated from the *Brassicaceae* family (Fahey et al., 2002). GLS share a consistent structure (Figure 5) containing a β-thioglucose unit linked to the carbon of a sulfonated oxime unit; but differ in the variable side chain (R) that are derived from amino acids (Sorenson, 1990). The R-substituent may be an alkyl or alkenyl side chain with various substituent options (hydroxyl group or sulfur). In addition, the R-substituent may also be an aromatic or heteroaromatic group. The side chain and the sulfate group have anti-stereochemical configuration. Since the 1970s, many studies have focused on the beneficial biological effects of GLS, and their breakdown products, on human and animal nutrition (Cartea and Velasco, 2008).

Figure 5. Common glucosinolate structure (Davidson et al., 2001).

Rapeseed has three main structural classes of GLS (Figure 8). These structures can be aliphatic (e.g. alkyl, alkenyl, hydroxyalkenyl, w-methylthioalkyl), aromatic (e.g. benzyl, substituted benzyl) or heterocyclic (e.g. indolyl) (Sorenson, 1990). Aliphatic, indole and aromatic side chains are derived from methionine, tryptophan and phenylalanine respectively. The chemicals derived from methionine, valine, isoleucine, leucine, or alanines are classified as aliphatic GLS. Those derived from tryptophan are called indole GLS and those derived from phenylalanine or tyrosines are aromatic (Sorenson, 1990). Because of the sulfate group, GLS occur in nature as anions and have strong acidic properties. These compounds can be counteracted by cations, potentially potassium being one of the most abundant cations in plant tissues. Hydrophilic properties are a characteristic of all GLS because of the sulfate group and thioglucose moiety.

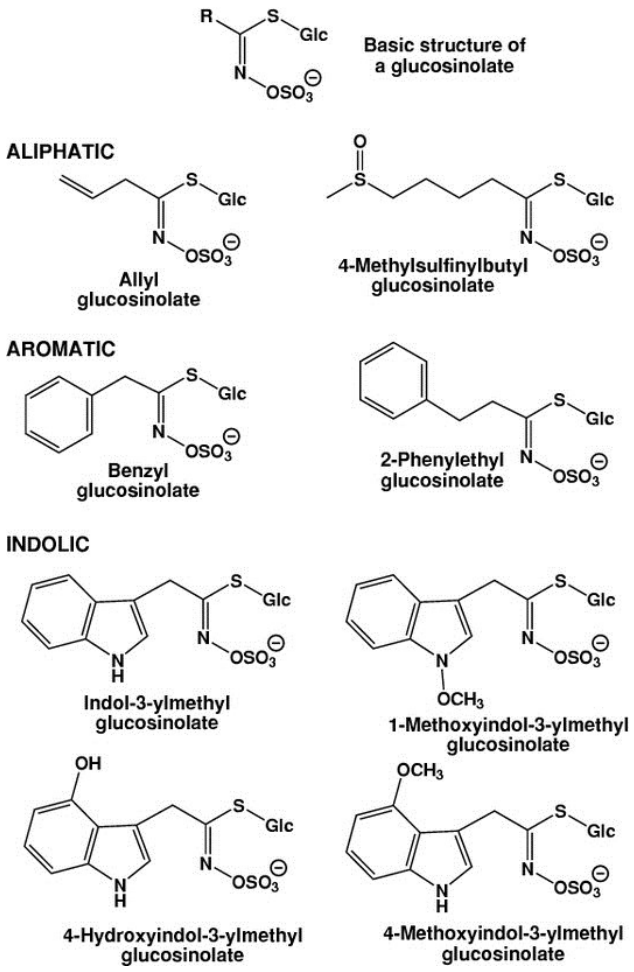

Figure 6. The three main structural classes of glucosinolates (aliphatic, aromatic, indolic) (Sorenson, 1990).

Glucosinolate Environmental Effects

Different plant parts contain different GLS concentrations and also have different GLS profiles. The adaptive significance of such differences is unclear, although high GLS concentrations tend to occur in rapidly growing plant parts such as shoot and root tips and may be associated with protection against damage by herbivores (Sang et al., 1983). The GLS and their breakdown products are thought to play an important role in general plant defense mechanism against fungal infection and non-adapted herbivores. However, in some instances

these compounds are also involved in host-plant recognition by specialized pests (Louda and Mole, 1991). For example, leaf-surface GLS can act as oviposition (i.e. egg-laying) stimulants for *Brassica sp*. adapted insects (e.g. cabbage and turnip root flies) (Hopkins et al., 1997). The volatile isothiocyanates produced by GLS breakdown can also attract pests to their host plants (Pivnick et al., 1992).

A number of environmental factors may also affect GLS profiles and concentrations; for example, increased concentrations of sulfate in soil solution increase GLS concentrations in both leaves and seed while increased soil nitrogen status has the opposite effect of reducing GLS concentration (Mailer, 1989). The effects of water availability on GLS concentrations in crucifers are unclear with some studies indicating a negative correlation between water supply and GLS concentration and others showing no response. Decreased light supply has also been shown to increase GLS concentrations in the plant (Blua et al., 1988). GLS content in plant vary with season. Autumn harvested foliage generally contains higher GLS than that occur in winter harvest (van Doorn et al., 1998; Agebirk et al., 2001). The water stress during vegetative or pod-filling stage increases the GLS content in the seed (Jensen et al., 1996).

Glucosinolate Biosynthetic Pathways

The general biosynthetic pathways of GLS shows that aliphatic GLS are derived from methionine, indole GLS are derived from tryptophan and aromatic GLS are derived from tyrosine or phenylalanine (Schonhof et al., 2004). In addition, a substrate-enzyme dependent route evolved in the core structure formation of the three major classes of GLS was classified into two main groups depending on the stage of the biosynthesis they control (Figure 7). The first group; cytochromes P450 belonging to the CYP79 family are responsible for catalyzing the conversion of amino acids to aldoximes, which will be converted into the corresponding aci-nitro

compound by the aid of the second group of enzymes CYP83 (Graser et al., 2001; Halkier and Gershenzon, 2006; Mewis et al., 2006). This is followed by the formation of a thiohydroximic acid by a C-S lyase, after which desulfoglucosinolate formation is catalyzed by S-glucosyl transferase, and finally the formation of GLS by sulfotransferase. The last three enzymes involved in the core structure formation are common for all classes of GLS (Windsor et al., 2005; Zang et al., 2009). The last phase in GLS biosynthesis is the side-chain modifications that involve oxidation, hydroxylation and methoxylation, which are under genetic and environmental control (Mithen, 2001).

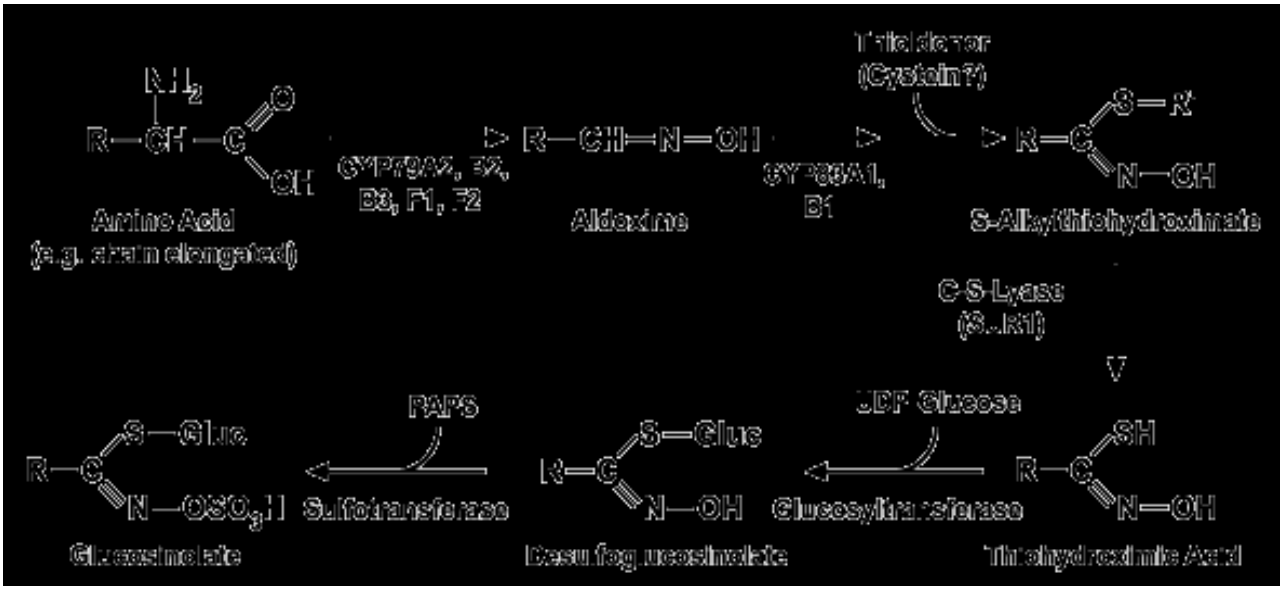

Figure 7. Glucosinolate biosynthetic pathway (Halkier and Gershenzon, 2006).

In the biosynthetic pathway of aliphatic GLS, methionine can undergo several elongation cycles for the addition of one methylene group at a time before it can enter the pathway for the formation of the GLS core structure (Textor et al., 2007). As in aliphatic GLS, phenylalanine may undergo a chain elongation step before the core structure formation of aromatic GLS.

CYP79A2 and CYP79B3 control aldoximes derived from phenylalanine and tryptophan for the synthesis of aromatic and indolic GLS, respectively. CYP83B1 catalyzes the conversion of aromatic and indolic aldoximes into the corresponding GLS (Halkier and Gershenzon, 2006; Windsor et al., 2005).

Glucosinolate Hydrolysis

Plants containing GLS possess thioglucosidase activity known as myrosinase, which hydrolyzes the glucose moiety on the main skeleton (Andersson et al., 2009). GLS and myrosinase are segregated in intact plants. They are chemically very stable, unless they come in contact with catalytic enzyme myrosinases, which are accumulated in different parts of cellular compartments. GLS themselves are biologically inactive, but their degradation products are active and known for their diversified biological effect. If the plant tissue and cells are processed, damaged and/or stressed, in the presence of water, myrosinases catalyse the hydrolytic cleavage of the thioglucosidic bond (Figure 8).

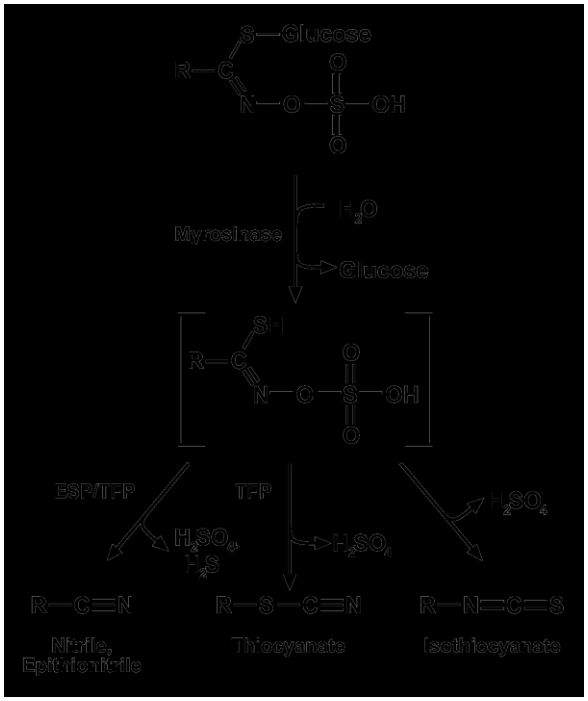

Figure 8. Glucosinolate hydrolytic pathway (Halkier & Gershenzon, 2006).

This cleavage step results in an organic aglucone moiety and includes equimolar amounts of glucose and potassium bisulfate. The remaining aglucones are not stable and are quickly converted to a thiocyanate, isothiocyanate or a nitrile, depending on the substrate, pH, and availability of ferrous ions (Fenwick et al., 1983; Mithen et al., 2000; Finiguerra et al., 2001; Bernardi et al., 2003). At physiological pH, isothiocyanates are the major product. The nature of the hydrolysis products is dependent on factors such as pH, temperature, metal ions, protein cofactors (i.e. epithiospecifier protein), and the properties of the side chain.

This myrosinase-GLS system may also be considered a part of the plant defense system against herbivores and pathogens, with at least six different types of these enzymes found in *A. thaliana*. In addition, myrosinase activity was very sensitive to ascorbic acid concentration (with maximum activity in the range of 0.7-1 mM), pH (4-7), temperature, and high salt concentrations (Finiguerra et al., 2001). This may indicate different functions of similar enzymes in the plant, in order to ensure plant fitness in different environments.

Rapeseed Glucosinolate Content and Composition

RSM contains three major GLS; progoitrin, gluconapin, and glucobrassicanapin (Table 6). The content and distribution of GLS varies among different varieties and origin. The RSM originated in Indian sub-continent contains chiefly gluconapin (Tyagi, 2002) while RSM from Europe and other temperate countries contain mainly progoitrin, 4-OH glucobrassicin and gluconapin (Mabon et al., 2000; Leming et al., 2004). The RSM from the decade of the 1980s had GLS ranging from 125 to 207 μmol/g, with mean value of 166 μmol/g dry oil-free meal; however, genetic manipulation produced low-GLS rapeseed varieties that contained 38 μmol/g GLS, ranging from 9 to 69 μmol/g dry oil-free meal in France (Bourdon and Aumaitre, 1990).

Table 6. Major glucosinolates found in *B. napus* and *B. campestris* rapeseed meals (Bell, 1984).

Glucosinolate	Semi-systemic name	R
Progoitrin	2-OH-3-butenyl-	CH_2=CH-CHOH-CH_3
Gluconapin	3-butenyl-	CH_2=CH(CH_2)$_2$
Glucobrassicanapin	4-pentenyl-	CH_2=CH(CH_2)$_3$
Napoleiferin	2-OH-4-pentenyl-	CH_2=CH-CH_2-CH-CH_2-OH
Glucobrassicin	3-indolyl-methyl-	$C_{16}H_{20}N_2O_9S_2$
Neoglucobrassicin	1-methoxy-3-indoly-methyl	$C_{17}H_{21}N_2O_{10}S_2^-$

Glucosinolate Importance to Humans

GLS have long held the interest of human society because of their presence in staple *Brassicaceae* vegetables (cabbage, broccoli) and condiments (mustard, horseradish). The distinct flavor associated with these foods is primarily the resulting products of isothiocyanate hydrolysis. Indole GLS and those with alkenyl R groups are especially known for causing bitterness (Engel et al., 2002). *Brassica* cultivars are finding increased use in bio-fumigation; which harvested plant material is incorporated into agricultural soils to suppress pathogens, nematodes, and weeds. GLS hydrolysis products are assumed to be the active agent in these treatments (Brown and Morra, 1995; Vaughn et al., 2005).

In the past decade, certain GLS have been identified as potent cancer-prevention agents in a wide range of animal models due to the ability of certain hydrolysis products to induce phase II detoxification enzymes, such as quinine reductase, gluthathione-S-transferase, and glucuronosyl transferases (Holst and Williamson, 2004; Keum et al., 2004). Sulforaphane, an isothiocyanate derivative of 4-methylsulfinyl-butyl GLS (found in broccoli), may prevent tumor growth by blocking cell cycles and promoting apoptosis (Zhang et al. 1992; Keum et al. 2004; Lund, 2003). In addition, sulforaphane exhibits potential for treating *Helicobacter pylori*-caused gastritis and stomach cancer (Fahey, 2002). Epidemiological studies have reported that the

isothiocyanates have positive effects against bladder, colon and lung cancers (Cartea and Velasco, 2008).

The effect of boiling, blanching and steaming on the levels of GLS, phenols, anthocyanins and antioxidant parameters in cauliflowers, cultivated under the same environment conditions and processed immediately after harvest, have been studied (Volden et al., 2009). It was observed that the least influence on these phytochemicals was with steaming and the biggest influence when boiled. The loss of a large fraction of GLS and antioxidants resulted in total GLS levels being reduced by 55 and 42% for boiling and blanching respectively, compared to only 19% by steaming. Other compounds were similarly affected, but to a lesser effect than GLS (Song and Thornalley, 2007).

Animal Effects Due to Glucosinolate Hydrolysis

The degree of adverse effect of dietary GLS depends on the level and compositions of GLS and their breakdown products. Different animal species have varying GLS tolerance capabilities and at higher levels, GLS may even cause mortality. Isothiocyanates have been found to be responsible for the bitterness in animal feed diets and the nitrile group has demonstrated health degrading animal effects (Mithen et al., 2000; Hill, 1991; Tanii et al., 2004). Iodine availability to the thyroid may be disrupted by thiocyanates, thiourea and oxazolidithione (Wallig et al., 2002). Other known negative effects of GLS metabolites include: goitrogenecity (Schone et al., 1993; Wallig et al., 2002), mutagenecity, hepatotoxicity and nephrotoxicity (Zang et al., 1999; Tanii et al., 2004). Research suggests that the adverse influence of dietary GLS on animal growth and production may be correlated to an endocrine disturbance induced by anti-nutritional factors (Ahlin et al., 1994).

The reduced feed intake by animals with diets that include GLS is due mostly to the presence of the bitter tasting sinigrin and progoitrin GLS (Fenwick et al., 1982). These reduced intakes often result in animal growth depression, especially in diets containing elevated GLS concentrations (Hill, 1991). Progoitrin, a non-bitter GLS in its natural form, is known to produce a more profound bitter taste compared to sinigrin, after the progoitrin is degraded (Doorn et al., 1998). Gluconapin is also considered a bitter GLS, but its impact on the diet intake is related to its content rather than its hindrance to animal preference (Heaney and Fenwick, 1980). These toxic and anti-nutritional effects have limited the use of seed meals in human diets and animal feed (Sorenson, 1990). The negative effects of GLS have been the basis for research targeting low GLS contents in some *Brassica* crops (Downing and Robbelen, 1989).

Rats and mice appear to be adversely affected (e.g. decreased food intake and growth depression) when the GLS content in their diets exceeds 4.4 mol/g (Vermorel et al., 1988) and when above 6.6 mol/g, poor gain and increased thyroid weight is observed (Wallig et al., 2002). Young pigs with total GLS diets of 2.79 mol/g experience reduced feed intake and growth (Corino et al., 1991). Extreme growth depression is observed in pig diets exceeding levels of 7 mol/g and diets containing total GLS levels above 9 mol/g have resulted in induced iodine deficiency, induced liver and thyroid hypertrophy, and reduced bone and serum zinc content and serum alkaline phosphatase (Mawson et al., 1994). In addition to growth depression, the toxicity has been shown to enlarge the liver and lower red blood cell counts in pigs (Schone et al., 1997). Studies recommend total GLS pig diets to be kept below 2 mol/g dry matter and supplemented with iodine (1000 g/kg) (Opalka et al., 2001).

Lowered feed intake, impaired growth, and increased mortality occur in poultry with elevated GLS consumption and appear to be less severe in broilers compared to laying hens and

turkeys (McNeill et al., 2004). Broiler tolerance studies suggest GLS diets anywhere between 8.0 and 11.6 mol/g result in severe growth depression (Mawson et al., 1994). Red seabream experienced a 15% growth reduction when consuming GLS diets at 2.18 mmol/kg intake (Glencross et al., 2004). A lower tolerance was observed by rainbow trout when fed GLS diets containing 1.4–19.3 mmol GLS/kg (Burel et al., 2000).

Ruminants appear to have a higher tolerance to GLS compared to monogastric animals, but chronic feeding of dietary GLS can cause of goitrogenecity, elevated plasma levels and depressed levels of plasma thyroxin (Tripathi et al., 2001). Fertility depression has also been demonstrated in cows fed rapeseed diets containing 31 mol/g DM total GLS (Ahlin et al., 1994). Studies also suggest that ruminant diets of total GLS levels from 11.7 to 24.3 mol/g reduce feed intake and milk production in dairy cows (Laarveld et al., 1981).

Glucosinolate Detoxification and Supplementation

Numerous methods have been studied to reduce GLS and/or their related toxicity to animals. Techniques such as microwaving (2450-MHz for 2.5 min) and micronization (90s at 195ºC) have been administered to effectively inactivate myrosinase and reduce GLS content in RSM (Fenwick et al., 1986). Feeding of heat treated RSM as a sole protein supplement improved milk yield, protein output, and showed higher dietary nitrogen utilization in dairy cows (Shingfield et al., 2003) and improved growth performance in growing calves (Tripathi et al., 2001).

Feed extrusion (dry and wet) also has proven to be an effective technique for reducing RSM GLS. Dry extrusion reduced total GLS by 193-428 kg/g and as high as 670 kg/g for wet extrusion (Huang et al., 1995). Soaking soybean meal in water and copper sulfate has been found to reduce total GLS by 900 kg/g and improve growth, thyroid function, iodine status, serum zinc

content and alkaline phosphatase activity in pigs and broilers (Das and Singhal, 2005). Water extraction to remove GLS for RSM has proven to be a cost effective method for removing GLS as long as the dry matter loss is managed (Tripathi et al., 2000).

RSM myrosinase can be inactivated using a 10 day solid state fermentation under aerobic conditions with *Rhizopus oligosporus* and *Aspergillus* species (Vig and Walia, 2001) and heat treatments (Bille et al., 1983). Iodine supplementation and copper sulfate treatments are common strategies to offset the deleterious animal effects of high GLS diets (Schone et al., 1993). The addition of 1000 g of iodine/kg (Schone et al., 2001) in pig diets containing 2 mol/g GLS to compensate the goitrogenic compounds that reduce iodine availability is recommended. Copper sulfate is added to help improve thyroid hormones and serum phosphatase in animals (Sihombing and Cromwell, 1974; Tripathi et al., 2001).

Although many techniques have been researched to manage/reduce GLS concentration in *Brassica* ingredients destined for animal diets, the procedures are not economically feasible for the feed industry. Alternative toxin management strategies include not utilizing the protein source in animal diets all together in fear of negative animal side-effects, or inclusion of meal in animal diets without screening for the toxin, in hope that the GLS concentration has no anti-nutritional effect.

Glucosinolate Analysis

GLS profiles in plants from different origins have been studied qualitatively and quantitatively for health, agricultural, economic and ecological purposes. Two main approaches have been used: the direct measurement of intact GLS and the indirect measurement of their derivatives. Intact GLS have been purified from plant material by reverse phase high performance liquid chromatography (HPLC) and identified by nuclear magnetic resonance

(NMR) and by mass spectrometry (MS) methods. These methods reported a high yield (>90%) for individual GLS (Song et al., 2006).

Indirect measurements of GLS derivatives produced by enzymatic hydrolysis (myrosinase and sulfatase enzyme) have been successful. Myrosinase enzymes are able to hydrolyze GLS and the enzymatically released glucose can be quantitatively measured, resulting in a total GLS concentration (Antonious et al., 2009). Sulfate hydrolysis of GLS, producing desulfated GLS, has been used widely for qualitative and quantitative analysis of individual GLS (Brown et al., 2003). Gas chromatography (GC) (Olsen and Sorensen, 1981), HPLC (Leoni et al., 1998) and NIRS (Font et al., 2005), were used for separation of desulfated glucosinolates, followed by comparative analysis using tandem-MS, ultra violet (UV) absorbance, NMR and retention time with pure standards for confirmation and identification (Agerbirk et al., 2001; Bellostas et al., 2007; Kiddle et al., 2001).

Quantification based on peak area of desulfated glucosinolates and comparison to peak area of internal or external standards can be accomplished by applying a relative response factor or calibration curves respectively, and a relative concentration for each individual compound has been obtained (Brown et al., 2003; Kim et al., 2009). Desulfated glucosinolates have been analyzed using HPLC-Atmospheric Pressure Chemical Ionization (APCI) /MS-MS methods (Griffiths et al., 2000). Desulfoglucosinolates were identified by application of fragmentation energy; the expected fragment weights were measured and used for qualitative analysis. The quantification method used was based on HPLC-APCI/MS and has the advantage of the ability to measure GLS at low concentrations, which was lower than the minimum quantification levels in the HPLC-UV method. The main disadvantage for utilizing a precise method like HPLC-

APCI is that calibration curves for each GLS must be prepared and these standards today are not commercially available.

The use of GC for separation and identification of trimethylsilyl and pertrimethylsilyl derivatives of intact and desulfated glucosinolates respectively has been studied (Olsen and Sorensen, 1981). The need for a derivatization step prior to analysis is not suitable for heat sensitive compounds. Moreover, the poor separation of GLS with a methylsulfinyl side chain makes the HPLC method more popular as both methods showed the same accuracy and precision.

The ability to rapidly measure total glucosinolates in oil extracted rapeseed meal could greatly improve antinutritional animal feed raw material screening, ultimately improving animal health and potentially reducing economic losses for livestock producers. Most of the rapeseed research conducted for rapid near infrared analysis has been done on the whole seed, with little emphasis on the defatted seed meal. Understanding if NIR or FT-NIR can accurately measure total glucosinolates in oil extracted rapeseed meal is a viable area of study.

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PAPER 1. GLUCOSINOLATE PRESERVATION IN STORED RAPESEED MEALS

Abstract

The objective of this study was to determine the storage stability of total glucosinolates (GLS) of various *Brassica* meals. Because GLS are being considered for potential biopesticide application and preventative cancer strategies, understanding the effects of storage conditions on GLS concentration may prove beneficial. The seed meals were stored at temperatures of -20, 4 and 22 ^{o}C in paper and polyethylene bags. These temperatures were chosen because they are common laboratory storage conditions. GLS concentrations were measured every 3 months for a total of 18 consecutive months using high performance liquid chromatography. Decreases were observed in all treatments stored at 4 ^{o}C (in both polypropylene and paper bags), and to the greatest extent in samples stored within paper bags. Relative humidity was elevated (above 80%) at this temperature, promoting visually obvious fungal growth in the paper bags. GLS reduction was observed in mechanically processed samples stored at 4 ^{o}C in polyethylene bags with no noticeable fungal growth. GLS preservation within stored *Brassica* meals was possible for 18 months and possibly longer providing the seed meals are protected from exposure to moisture conditions that promote microbial growth and potentially endogenous myrosinase hydrolysis.

Introduction

Rapeseed ranks second in world-wide oilseed production (after soybean) and protein meals and contributes approximately 13% of the total oilseed and protein meal production in the world (Lennox and Beckman, 2011; USDA, 2013). Global production of rapeseed exceeds 60 million metric tons with the major producers being Europe, China, Canada, and India (USDA, 2013). Because of its global production scale, various production methods are utilized to process rapeseed/canola (*Brassica napus*) and mustards (*Brassica juncea*). The process of oil extraction

from rapeseed includes seed grading, drying, handling, seed cleaning/preparation, extraction, and processing of the oil (Salunkhe et al., 1992; Unger, 2011). Oil extraction can be categorized by two processes: with or without solvent extraction (Adams et al., 2006). If solvent extraction is not used, oil may be expelled from the seeds using mechanical pressing (Adams et al., 2006; Spragg and Mailer, 2007; Newkirk, 2009). Solvent extraction production involves numerous cooking and heating steps that help to deactivate enzymes like myrosinase and lipase in the rapeseed meal (RSM), but these steps are limited in mechanical processing.

Rapeseed and mustard crops are receiving increased interest because of their rotational and environmental benefits making them an excellent choice for increasing agricultural sustainability. These *Brassica* oilseeds are extremely competitive with most annual weed species, reducing the need for chemical weed control (Beckie et al., 2008). Allelochemicals produced by rapeseeds and mustard tissues also provide pest control benefits and are highly drought resistant (Brown and Morra, 1997). In addition, the production emphasis of biofuels as a petroleum substitute has increased global rapeseed production. Canola meal has commercial value as an animal feed, but rapeseed and mustard meal GLS levels may restrict meal utilization in animal feed.

At least 120 structurally different GLS have been identified in 16 different families of angiosperms (Fahey et al., 2001), including rapeseed and mustard. Although GLS themselves possess limited biological activity, enzymatic degradation by myrosinase results in the formation of a number of compounds (nitriles, isothyiocyanates, thiocyanates, etc). GLS are found in all plant parts, but they are most concentrated in the seed. Crushing the seed for oil removal produces a meal product that contains not only high concentrations of GLS, but preserves

enzyme activity such that water addition results in the formation of biologically active hydrolysis products unless the enzymes are deactivated by heat during processing (Brown et al., 1991).

Because GLS metabolites are being considered for potential agricultural application and medical uses, understanding GLS concentration effects during storage could prove beneficial. In order to study glucosinolates and to understand their shelf-life, it is imperative to understand whether or not GLS within the seed meals remain intact during different storage conditions and time periods. It is also valuable to understand if meal processing differences have varying effects on GLS stability in various RSM. The objective of this study is to investigate the potential total RSM GLS concentration change under various storage conditions for meals obtained by different meal processing techniques. The hypothesis of this study is that storage conditions will not affect total GLS concentration over 18 months in solvent extracted rapeseed samples. In contrast, it is believed that samples processed mechanically will show statistically significant decreases over the 18 month trial.

Material and Methods

Meal origination and sample preparation

A total of 5 kg of RSM was obtained from 6 different oilseed crushing locations within 12 hours of meal production. The samples were placed in sterile whirl pack bags and overnighted to a central laboratory in Elk River, MN. Upon arrival, the samples were stored and monitored in a freezer maintained at -20 ^{o}C until all samples were received from the various oilseed plants. Samples were stored between 2-5 days under these conditions. Four of the six RSM samples were processed under similar conditions (e.g. flake size, temperature, solvent retention) with hexane to remove the oil. These samples included: India Rapeseed Meal (IRM), China Rapeseed Meal (CRM), Canada Canola Meal (CCM), and India Mustard Meal (IMM). India Rapeseed

Meal Mechanical (IRMM) and Canada Canola Meal Mechanical (CCMM) were processed with a mechanical press to remove the oil. Each sample was split with a Gilson SP1 sample splitter (Lewis Center, OH) into 36, 100 g subsamples.

Seed meal storage conditions

The seed meals were stored in two different containers (fully air-permeable paper bags and sealed polyethylene bags) at -20 ± 2, 4 ± 2, and 22 ± 3 ^{o}C. Each temperature/container treatment was replicated 6 times. The trial lasted 18 months with sampling and total GLS analysis (via HPLC) performed at the beginning of the trial and every third month. Containers were removed from the designated temperature controlled environments and exposed to room air only during the sampling process every 3 months.

Glucosinolate HPLC analysis

Meal analysis for total GLS was conducted in triplicate by HPLC according to ISO method 9167-1 (ISO, 1992). Each sample (200 ± 0.1 mg) was transferred into a 6 mL polypropylene tube and weighted to the nearest 0.1 mg. In order to extract the GLS, the tubes were placed into a water bath set at 75 °C and left for one minute. Two mL of boiling methanol solution (70 % v/v) was then added to each tube in order to inactivate the myrosinase. Then, 200 µL of 20 mM internal standard solution (Indofine Chemical Company, USA) was added to the tube. The test tube contents were constantly agitated and heated to 75 ^{o}C for 10 minutes. The contents were centrifuged at 5000 RCF for 3 minutes. The supernatant was then transferred to another tube. The extraction step was repeated by adding 2 mL of boiling methanol solution to the residue and reheated for 10 minutes in a water-bath set at 75 ^{o}C with constant agitation. The contents of the tubes were then centrifuged for 3 minutes at 5000 RCF and the supernatant was added to the respective supernatant liquid acquired from the original extraction. Finally, the

volume of the extracts were brought to 5 mL with ultrapure water and mixed. After the extracts were prepared, they were concentrated using ion exchange chromatography.

Vertical Pasteur pipettes (150 mm long), for each extract, were placed on a stand leaving a volume of 1.2 mL above the neck. Glass wool plugs were placed in the neck of each pipette. A total of 0.5 mL of well-mixed suspension of ion-exchange resin (DEAE Sepharose CL-6B, Pharmacia Biotech, Sweden) was added to each pipette and allowed to settle. The pipettes were then rinsed with 2 mL of imidazole formate (Wako Company, USA) solution (6 mol/L) and twice with 1 mL portions of ultrapure water.

The GLS extract (1 mL) was purified by passing it over the ion-exchange column. After the extract solvent passed through the column, two 1 mL portions of sodium acetate buffer (0.02 mol/L at pH 4.0) was added to the column and allowed to drain after each addition. Desulfatation was achieved by adding 75 μL of purified sulfatase (EC 3.1.6.1, Sigma, USA) solution (1 mL of purified sulfatase to 10 mL one-mark volumetric flask) to the column and allowed to act on the GLS overnight at ambient temperatures. Desulfated GLS was eluted with two 1 mL ultrapure water portions, allowing the water to drain after each dilution into a tube. The eluants were mixed and stored in a dark freezer at -18 ^{o}C before HPLC analysis.

A total of 25 μL of the desulfoglucosinolate solution was injected into a Waters 600 HPLC instrument (Milford, MA) equipped with a Waters model 486 UV tunable absorbance detector (Milford, MA) at a wavelength of 229 nm. The desulfoglucosinolates were separated using a C18 column (CAPCELL PAK C18 Type: C18 AG 120 A; 4.6 mm × 150 mm, 5 μm) with a flow rate of 0.5 ml/min at 30° C. Elution of desulfoglucosinolates from HPLC was performed by a gradient system of water (A) and acetonitrile/water (25:75, v/v, B). The total running time was 45 min with a gradient as follows: 100% A and 0% B for 5 min, then in 35 min

to 0% A and 100% B and in 5 min back to 100% A and 0% B. Individual GLS were identified in comparison with the retention time of the standards (Table 7). Total and individual GLS were expressed as μmol/g of dry matter (APPENDIX A).

Table 7. Desulfoglucoinolate response factors used during HPLC analysis of glucosinolates.

	Desulfoglucosinolates	**Response Factor**
1	Desulfoglucoiberin	1.07
2	Desulfoprogoitrin	1.09
3	Desulfoepi-progrotrin	1.09
4	Desulfosinigrin	1.00
5	Desulfogrlucoraphanin	1.07
6	Desulfogluconapoleiferin	1.00
7	Desulfoglucoalyssin	1.07
8	Desulfogluconapin	1.11
9	Desulfo-4-hydroxyglucobrassicin	0.28
10	Desulfoglucobrassicanapin	1.15
11	Desulfoglucotropaeolin	0.95
12	Desulfoglucobrassicin	0.29
13	Desulfogluconasturtin	0.95
14	Desulfo-4-methoxyglucobrassicin	0.25
15	Desulfoglucobrassicin	0.20
16	Other desulfoglucosinolatees	1.00

HPLC method validation

A certified reference material, Rapeseed ERM-BC367, was obtained from the European Commission and was analyzed in triplicate on four consecutive days to determine the accuracy of the assay. The certified range for the test sample was 99 ± 9 μmol/g total GLS. In addition, two RSM varieties (Canada Canola Meal, 4.65 μmol/g total GLS; India Mustard Meal, 143.55 μmol/g total GLS) were each split into 24 individual 50 g subsamples with a Gilson SP1 sample splitter (Lewis Center, OH). Six of the subsamples for each variety were analyzed over four consecutive days to demonstrate the robustness and repeatability of the method.

Statistics

For HPLC analysis, the content of each GLS, expressed in micromoles per gram (μmol/g) of dry matter of the product, was equal to:

$$(A_g/A_s) \times (n/m) \times K_g \times (100/100\text{-}w)$$

Where

A_g is the peak area, in integrator units, corresponding to desulfoglucosinolates;

A_s is the peak area, in integrator units, corresponding to desulfosinigrin;

K_g is the response factor (Table 7) of desulfoglucosinolates;

m is the mass, in grams, of the test portion;

n is the quantity, in micromoles, of internal standard added;

w is the moisture and volatile matter content, expressed as a percentage of mass, of the test sample.

The total GLS content, expressed in micromoles per gram (μmol/g) of dry matter of the product, was equal to the sum of the contents of each GLS.

Microsoft Excel 2010 was used to calculate the HPLC method accuracy and reliability, by calculating the test average, percent error and percent relative standard deviation (%RSD). The same Excel program was used to calculate the regression equations and coefficient of determination (R^2) for total GLS, at different temperatures and storage conditions, for *Brassica* meal samples over time. In addition, differences within a treatment over time and between b bag/temperature treatments for each sample type were compared by two-way analysis of variance (ANOVA) using Microsoft Excel 2010. Probability (p) values of <0.05 were considered significant.

Results and Discussion

The HPLC cross check sample obtained from the European Commission averaged 98.5 μmol/g with a percent error of 0.58 % and a %RSD of 1.4. The average result was within the certified test range (99 ± 9 μmol/g total GLS) for the sample. The %RSD for the repeatability study ranged from 1.26 for the canola meal standard to 2.25 for the India RSM sample. These results were acceptable according to the ISO 9167-1 standard and similar to studies utilizing the same assay (ISO, 1992; Velasco and Baker, 1998; Hom et al., 2007; Embaby et al., 2010).

The initial average total GLS concentration for the six samples chosen for the study ranged from 6.10 to 138.04 μmol/g (Table 8). The individual GLS concentrations varied between *Brassica* meal samples. The rapeseed and mustard meal samples originated from India (IRSM, IMM) were mostly composed of the allyl and 3-butenyl GLS, while the Canada Canola Meal (CCM) and China RSM (CRSM) samples mostly contained the 3-butenyl and 2-OH-3 butenyl GLS. All of the individual GLS values for the samples were within 1 standard deviation of their mean.

Table 8. The initial individual and total glucosinolate concentrations (µmol/g) for various *Brassica* meals.

Aliphatic
Aromatic
Indole
nd = non-detect

Rapeseed types	allyl (sinigrin)	3-butenyl (gluconapin)	2-phenylethyl	2-OH-4-pentenyl (napoleiferin)	2-OH-3-butenyl (progoitrin)	methylthiopentenyl	methylthiobutenyl	4-OH-benzyl (sinalbin)	4-pentenyl (glucobrassiconapin)	3-methylindolyl (glucobrassicin)	4-hydroxyglucobrassicin	Total
IRSM	15.10	54.33	0.37	0.06	0.41	0.00	7.40	nd	0.82	nd	1.07	79.56
CRSM	0.75	6.05	0.12	0.19	7.86	0.09	0.21	nd	0.86	0.09	0.43	16.65
IMM	29.34	102.15	0.63	0.09	0.71	nd	0.07	nd	1.51	nd	3.54	138.04
IRSMM	22.00	82.89	0.55	0.06	0.70	nd	1.50	nd	1.32	dn	1.91	110.93
CCMM	nd	2.05	0.05	0.72	6.76	0.06	0.14	nd	0.68	0.18	2.34	12.98
CCM	nd	2.25	nd	nd	2.52	nd	nd	0.23	nd	nd	1.10	6.10

Total GLS concentration changed over 18 months for the *Brassica* meal samples stored in paper bags at -20, 4, and 22 ºC (Figures 9, 10, and 11 respectively). The largest concentration reduction over 18 months was in samples stored in paper bags at 4 ºC for the two mechanically processed *Brassica* meals (Table 9). The total GLS concentration decreased by 25% for India RSM samples that were processed mechanically over the 18 months while the Canada Canola Meal Mechanical samples decreased by 50% over 18 months. India mustard meal, India RSM, and China RSM obtained from conventional processing also had total GLS reduction over the 18 month trial and all samples had noticeable mold growth.

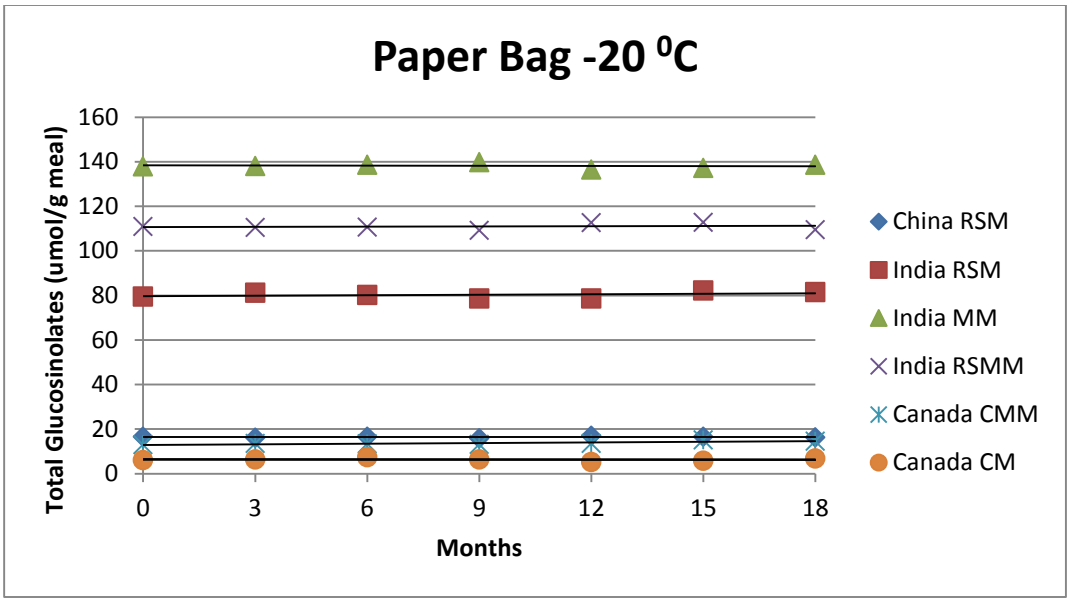

Figure 9. Total glucosinolate concentration in various *Brassica* meals stored at -20 °C for 18 months within a paper bag.

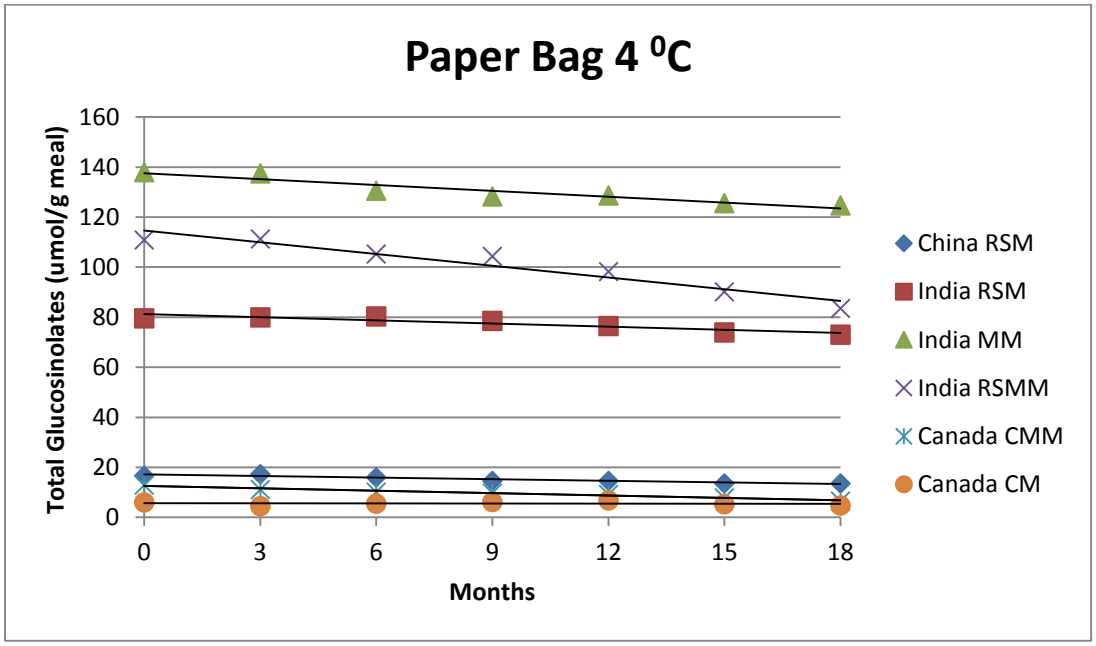

Figure 10. Total glucosinolate concentration in various *Brassica* meals stored at 4 °C for 18 months within a paper bag.

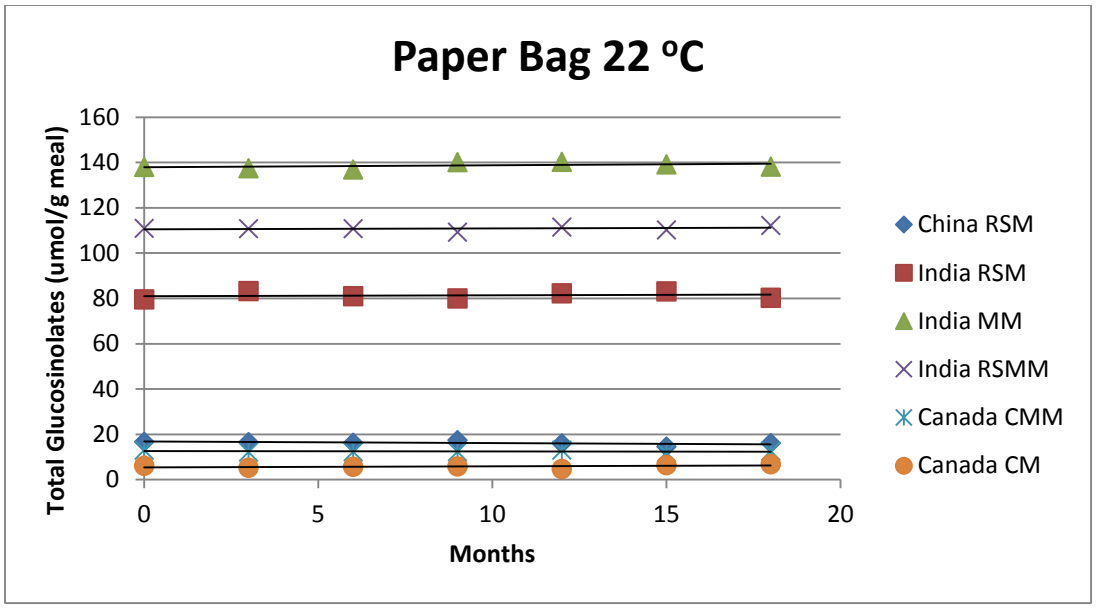

Figure 11. Total glucosinolate concentration in various *Brassica* meals stored at 22 °C for 18 months within a paper bag.

Table 9. Regression equation and coefficient of determination for total glucosinolates for *Brassica* meal samples stored in paper bags over 18 months.

Rapeseed Meal	**Paper Bag -20°C**	**Paper Bag 4°C**	**Paper Bag 22°C**
China Rapeseed Meal	$y = 0.003x + 16.443$ $R^2 = 0.0031$	$y = -0.2131x + 17.129$ $R^2 = 0.8869$	$y = -0.0693x + 16.831$ $R^2 = 0.2687$
India Rapeseed Meal	$y = 0.0787x + 79.588$ $R^2 = 0.1241$	$y = -0.4187x + 81.205$ $R^2 = 0.8491$	$y = 0.035x + 81.022$ $R^2 = 0.0224$
Canada Canola Meal	$y = -0.0111x + 6.4025$ $R^2 = 0.0123$	$y = -0.0142x + 5.7432$ $R^2 = 0.0129$	$y = 0.0426x + 5.4464$ $R^2 = 0.1446$
India Mustard Meal	$y = -0.0181x + 138.35$ $R^2 = 0.1241$	$y = -0.7837 + 137.56$ $R^2 = 0.8995$	$y = 0.0867x + 137.85$ $R^2 = 0.1821$
India Rapeseed Meal Mechanical	$y = 0.0301x + 110.65$ $R^2 = 0.0031$	$y = -1.5651x + 114.64$ $R^2 = 0.9321$	$y = 0.041x + 110.44$ $R^2 = 0.0798$
Canada Canola Meal Mechanical	$y = 0.0963x + 12.91$ $R^2 = 0.6102$	$y = -0.3212x + 12.591$ $R^2 = 0.9685$	$y = -0.011x + 12.581$ $R^2 = 0.0.31$

Total GLS concentration changed over 18 months for the *Brassica* meal samples stored in polyethylene bags at -20, 4, and 22 °C (Figures 12, 13, and 14, respectively). Similar to the

study conducted with paper bags; the largest 18 month reduction in GLS was in samples stored in polyethylene bags at 4ºC for the two mechanically processed *Brassica* meals. The GLS in India RSM samples that were processed mechanically decreased by 12% over 18 months and the Canada Canola Meal Mechanical samples decreased 37%. The GLS content in meal samples stored in polyethylene bags at the other temperatures were not affected by storage conditions (Table 10).

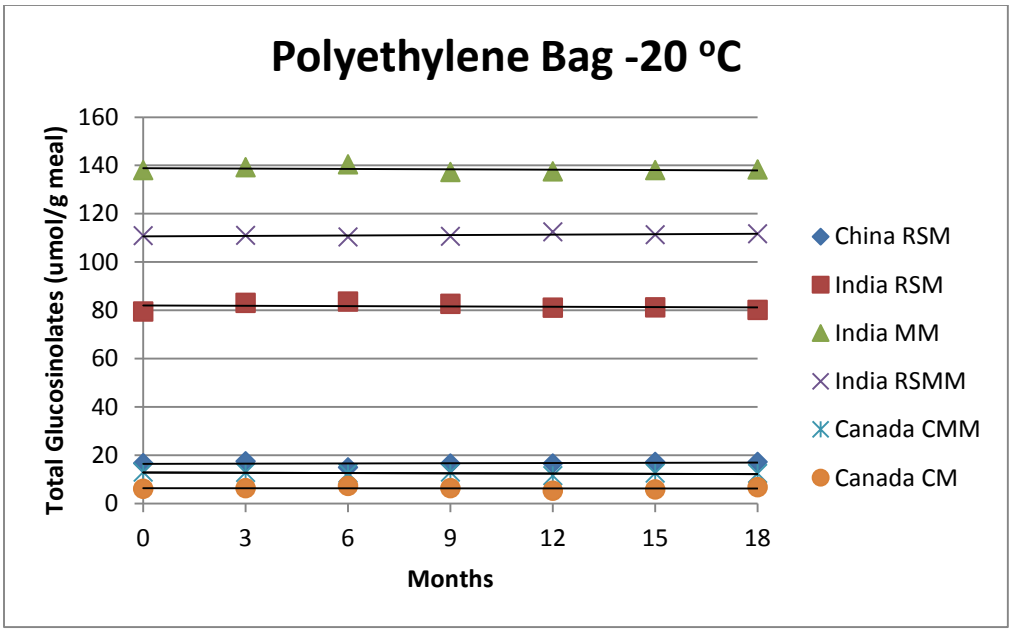

Figure 12. Total glucosinolate concentration in various *Brassica* meals stored at -20 ºC for 18 months within a polyethylene bag.

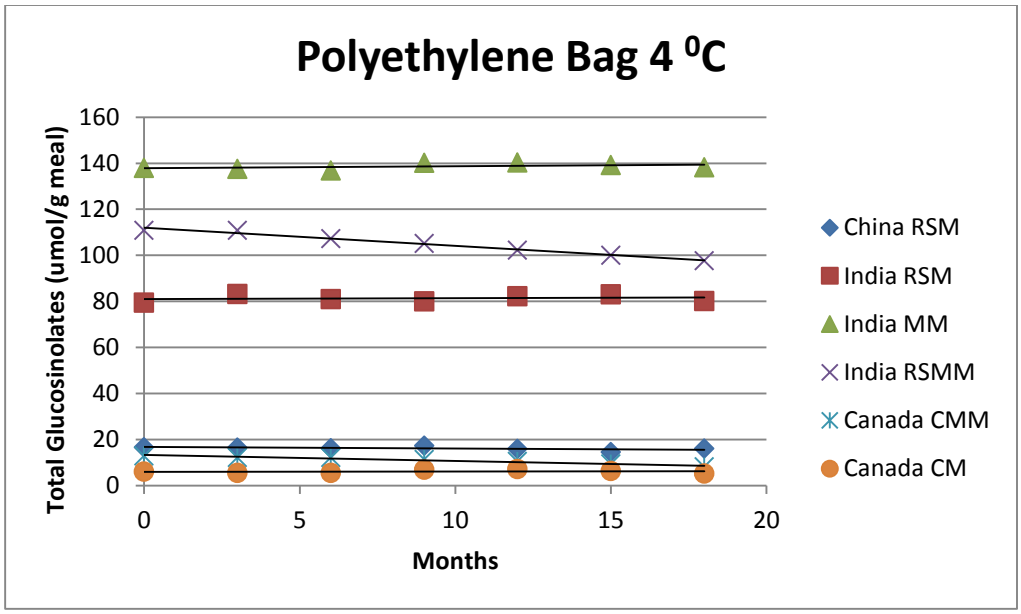

Figure 13. Total glucosinolate concentration in various *Brassica* meals stored at 4 °C for 18 months within a polyethylene bag.

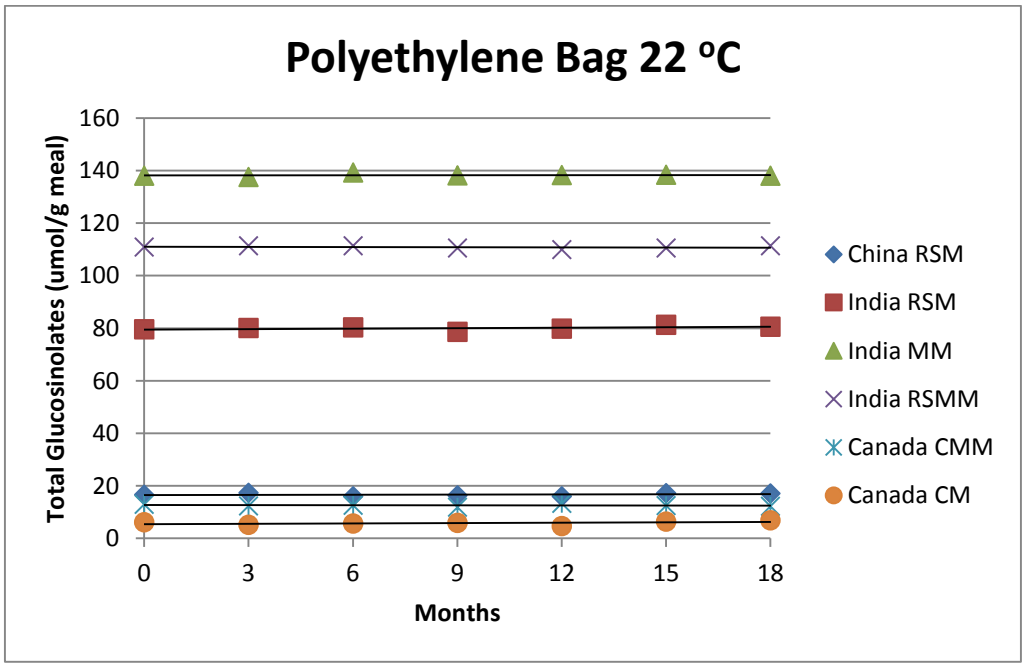

Figure 14. Total glucosinolate concentration in various *Brassica* meals stored at 22 °C for 18 months within a polyethylene bag.

Table 10. Regression equation and coefficient of determination for total GLS for *Brassica* meal samples stored in polyethylene bags over 18 months.

Rapeseed Meal	Polyethylene Bag -20 °C	Polyethylene Bag 4 °C	Polyethylene Bag 22 °C
China Rapeseed Meal	$y = -0.0326x + 16.388$ $R^2 = 0.0623$	$y = -0.0693x + 16.831$ $R^2 = 0.2687$	$y = 0.0144x + 16.546$ $R^2 = 0.028$
India Rapeseed Meal	$y = -0.0508x + 82.093$ $R^2 = 0.0474$	$y = 0.035x + 81.022$ $R^2 = 0.0224$	$y = 0.0607x + 79.469$ $R^2 = 0.2052$
Canada Canola Meal	$y = -0.0111x + 6.4025$ $R^2 = 0.0123$	$y = 0.0105x + 6.04$ $R^2 = 0.0086$	$y = 0.0426x + 5.4464$ $R^2 = 0.1446$
India Mustard Meal	$y = -0.0525x + 138.88$ $R^2 = 0.0975$	$y = 0.0867x + 137.85$ $R^2 = 0.1821$	$y = 0.0061x + 138.16$ $R^2 = 0.2052$
India Rapeseed Meal Mechanical	$y = 0.0623x + 110.65$ $R^2 = 0.297$	$y = -0.7901 + 112$ $R^2 = 0.9824$	$y = -0.0227x +$ 111.03 $R^2 = 0.0696$
Canada Canola Meal Mechanical	$y = -0.0385x + 12.915$ $R^2 = 0.259$	$y = -0.2575x + 13.32$ $R^2 = 0.9507$	$y = -0.0148x +$ 12.689 $R^2 = 0.0395$

Two-way analysis of variance (ANOVA) was used to determine if the GLS reduction over time for some treatments was significant and to test for significant differences between treatments for each sample type. Even though changes were noticed for the non-mechanically processed samples, the means for these treatments were not statistically significant, with the exception of Indian Rapeseed Meal (Table 11). The two mechanically processed samples indicated significant differences, suggesting that at least one of the group means (i.e. 4 °C storage conditions) was significantly different. Post hoc comparisons using the Fisher LSD test revealed that the mechanically processed samples stored in both paper and polyethylene bags at 4 °C showed a significantly greater reduction overtime compared to the other treatments.

Table 11. Two-way ANOVA comparing changes over time and between treatments of oilseed types.

	Over time			Between treatments		
Rapeseed Meal Type	F	F-critical	p-value	F	F-critical	p-value
Canada Canola Meal	1.13	2.53	0.37	0.35	2.42	0.90
Canada Canola Mechanical	11.11	2.53	4.04×10^{-6}	1.68	2.42	0.16
Indian Mustard Meal	0.27	2.53	0.92	0.65	2.42	0.68
China Rapeseed Meal	0.80	2.53	0.56	1.36	2.42	0.26
Indian Rapeseed Meal Mechanical	7.83	2.53	8.19×10^{-5}	1.88	2.42	0.12
Indian Rapeseed Meal	3.85	2.53	0.01	5.46	2.42	6.37×10^{-4}

GLS degradation is possible through two different pathways: enzymatic hydrolysis by endogenous myrosinase and microbial growth and metabolism (Fahey et al., 2001). Samples used in this study were processed mechanically (Canada Canola Meal Mechanical and India RSM Mechanical) or through solvent extraction (China RSM, India RSM, Canada Canola Meal, India Mustard Meal). The solvent extraction process involves a desolventization/toasting step, which includes direct steam application to the meal at temperatures above 110 ^{o}C. Processing at these temperatures inactivates the myrosinase enzyme and reduces GLS concentrations up to 65% in the meal (Klein-Hessling, 2007). Mechanical pressing only introduces frictional heat, which does not exceed more than 60 ^{o}C during processing, falling short of the required 90^{o}C temperature needed to completely inactivate myrosinase (Bagger et al., 1998). The active myrosinase could be the reason why the mechanically pressed RSM samples had greater total GLS reduction at 4 ^{o}C compared to solvent extracted samples. Previous research indicates that enzymatic hydrolysis by endogenous myrosinase can occur at 0.2 g H_20/g protein (Careri et al., 1980). Hydration isotherms indicate that this water content (0.2 g H_20/g protein) can be achieved by exposing enzymes to relative humidity near 80 %, which was possible in the 4 ^{o}C storage incubator (Lind et al., 2004).

All of the samples stored in paper bags at 4 ºC had visible fungal growth on the meal samples and had total GLS reduction. Equilibrium relative humidity relationships have been reported for RSM, showing that the largest meal water contents occur at the highest relative humidities and lowest temperatures (Jayas et al., 1988). Fungal growth in food products stored for 6 months or more is typically inhibited if the relative humidity is kept below 65-70 %, whereas relative humidity in the 4 ºC incubator may have routinely exceeded this lower limit (Pitt and Hocking, 1997).

GLS in the *Brassica* meals are readily available microbial substrates because seed crushing destroys plant cell membrane integrity releasing water-soluble anion. Microorganisms will exploit microenvironments where moisture is adequate for growth, facilitating product removal and thus theoretically enhancing GLS degradation. Even when endogenous myrosinase was deactivated because of processing, microbial growth and total GLS reduction occurred in the paper bags stored at 4 ºC. The reason for the GLS reduction might be related to the myrosinase activity expressed by a number of microorganisms that are capable of growing in *Brassica* meals including *Aspergillus, E. coli,* and *Fusarium* species (Smits et al., 1993; Ohtsuru et al., 1973).

Limiting exposure to air by storage in polyethylene bags prevented visibly noticeable fungal growth at all temperatures (including the samples stored at 4 ºC). Only samples stored in polyethylene bags at 4 ºC that exhibited noticeable total GLS reduction were the samples that likely contained active myrosinase. Periodic removal of the meals samples resulted in air and water vapor exposure that may have fostered microbial growth not obvious through visual examination or it may have stimulated endogenous myrosinase activity.

For the mechanically processed samples, the largest losses in individual GLS over time was experienced by the GLS that made up the majority of the total GLS concentration.

Specifically, for the mechanically processed Indian Rapeseed Meal, the 3-butenyl GLS appeared to be the only GLS that decreased overtime, and the same was true for the 2-OH-4 butenyl GLS mechanically processed canola meal. Aliphatic (i.e. 3 – butenyl, and 2-OH-4 butenyl) and aromatic GLS are known to be stable over time unless hydrolyzed by myrosinase (Fahey et al., 2001). Indole GLS are more unstable than aliphatic and aromatic GLS and can decompose spontaneously, without myrosinase (Kim and Jander, 2007). For *Brassica* meals, indole GLS are low in concentration and for this study did not result in a noticeable reduction.

Conclusion

The GLS losses occurring in *Brassica* meals stored in both bag types at 4 ^{o}C and relative humidity near 80% indicate that the primary requirement for maintaining GLS concentrations during sample storage is limiting water content so as to avoid microbial growth and prevent endogenous myrosinase activity. The objective of this study to investigate the potential total RSM GLS concentration change under various storage conditions for meals obtained by different meal processing techniques was met and the outcome was as expected. The storage conditions did not statistically affect total GLS concentration over 18 months in solvent extracted rapeseed samples. Also, as expected, the GLS from mechanically processed samples decreased over time at 4 ^{o}C and were statistically different compared to the other treatments.

The meal storage was possible up to 18 months and presumably longer provided that the relative humidity of the storage atmosphere inhibits obvious fungal growth, regardless of how the sample was processed. The type of container used for meal storage is only of consequence if relative humidity is high enough to support microbial growth on the seed meal. Both analytical chemists and biopesticide applicators need to consider these effects when assessing GLS

concentration and functionality. Other packing materials such as nylon bags could be an option for sample storage under high relative humidity.

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PAPER 2. GLUCOSINOLATE ASSESSMENT IN *BRASSICA* MEALS BY NEAR-INFRARED SPECTROSCOPY

Abstract

Glucosinolates (GLS) and their hydrolysis products are of great concern because they are responsible for both the beneficial and harmful properties of GLS-containing plants. The by-products produced from *Brassica* species oil processing are commonly used in animal feeds as a protein source and understanding the total GLS concentration in the meals is important to ensuring livestock health. The standard analytical techniques for determining GLS composition using conventional methods lead to high costs, labor inputs and delays, all of which affect both the availability of data and ability to make timely decisions. In contrast, near infrared spectroscopy (NIRS) has emerged as a rapid and cost effective technique of analysis for many agro-food products. The potential of NIRS for screening the total GLS content of various *Brassica* meals obtained globally and over multiple growing seasons was evaluated. Spectra data from 400 to 2500 nm were recorded on the various *Brassica* meal samples (186) at 2 nm intervals. A global calibration using the *Brassica* database was developed with a modified partial least squares regression (MPLS) of conventional laboratory analysis. The calibration preprocessing included the first derivative and standard normal variate data processing. The NIRS calibration for total GLS resulted in a coefficient of determination (R^2), standard error of the calibration (SEC), and a relative predictive determinant (RPD) of 0.96 and 6.05, and 6.32, respectively. An examination of the loadings of the equation for total GLS suggested that O-H groups of water, C-H combinations of the methylene groups and N-H groups of amides were the molecular associations most strongly used in modeling total GLS. The use of NIRS as a routine

analytical method for determining total GLS in *Brassica* meals destined for animal feeds has great potential.

Introduction

Glucosinolates (GLS) are secondary metabolites found in cruciferous plants that contain a side chain (R) and a sulfur atom linked D-pyranoid glucose (Shan et al., 2007). The structural diversity of this large group of compounds is due almost entirely to the different substituents possible at the side-chain position. The hydrolysis products of GLS are of great interest because they are responsible for many of the beneficial effects of plants containing GLS including antibacterial and antifungal properties, and potential cancer chemo-prevention (Rosa and Heaney, 1993; Shapiro et al., 2001). Sulforaphane, one of the degradation products of GLS, not only has anti-carcinogenic potential, but also produces flavor in cruciferous vegetables (Rosa et al., 1997). In contrast, the GLS in *Brassica* species have shown toxic and anti-nutritive effects in animals including goitrogenic effects and feed intake reduction (Tannii et al., 2004; Wilkinson, 1984).

Reverse phase HPLC quantitative analysis of desulfurized glucosinolates was established by Spinks et al., and is an official method for the International Organization of Standardization (ISO) and European Union (Spinks et al., 1984; ISO, 1992; Bertrand and Heinrich, 2000). Near-infrared spectroscopy (NIRS) is characterized by low molar absorptivities and scattering, which allows for evaluation of pure materials. The near infrared (NIR) region of the electromagnetic spectrum, once regarded as having little potential for analytical work, has now become one of the most promising areas for molecular spectroscopy. The arrival of inexpensive and powerful computers has contributed to the surge in NIR applications in several fields, including the medical and pharmaceutical fields and in traditional food/grain analysis (Lio et al., 2012).

The standard HPLC techniques for determining GLS composition using conventional methods are costly, require a high labor input, and create delays, affecting the availability of data and ability to make rapid decisions (Cozzolino, 2009). In contrast, the use of fast analytical techniques, such as NIRS, have many advantages since analysis can be carried out with a considerable saving of time, at a lower cost and without using hazardous chemicals.

Most research on NIRS for predicting *Brassica* GLS levels has focused on *B. napus* "double zero" (i.e. Canola) whole seeds (Biston et al., 1988; Williams and Sobering, 1993; Daun et al., 1994; Michalski and Krzymanski, 1988) and Indian mustard (*Brassica juncea*) whole seeds (Velasco and Becker, 1998; Font et al., 2005). In contrast, the GLS NIRS research for industry rapeseed and mustard meals are scarce and need further attention to determine if NIRS is a viable analytical technique for measuring total GLS in reduced oil *Brassica* meals. The objective of this study was to determine if a quantitative total GLS NIR calibration could be developed. A total GLS calibration can be developed with a relative predictive determinant (RPD) at 5 or above was hypothesized.

Materials and Methods

Plant material obtainment and preparation

The study consisted of evaluating 186 *Brassica* meal samples from different geographical regions (North America, Europe, and Asia) comprising multiple crop harvests. The samples represented various Cargill Inc., *Brassica* meal suppliers from 2012 and 2013 and the samples were compiled and retained (22 ^{o}C) in a laboratory in Elk River, MN (APPENDIX B). Suppliers submitted 150 g of sample, in sterile screw tight cups, to specified Cargill Animal Nutrition regional laboratories (located in United States, Italy, China, South Korea, and Philippines). These samples were then forwarded

weekly to the Elk River, MN laboratory for retention and future analysis by NIR and HPLC. The samples were mixed thoroughly by hand and split into three 50 g sub-groups. Two of the sample groups were ground to pass through a 1-mm screen using a cyclonic mill (Retsch GmbH & Company, Hann, Germany). The third sample was retained in its original state.

NIR spectra acquisition

One set of the ground meal samples were analyzed by a NIRSystems 6500 spectrophotometer (Foss-NIRSystems, Inc., Hilleroed, Denmark) equipped with a transport module, in the reflectance mode. Both visible and NIR spectra were collected utilizing lead sulfide (1100 - 2500 nm) and silicon (400 - 1100 nm) detectors. A total of 30 g of 1 mm ground sample was packed in the NIRS quarter cup made of quartz glass and the spectrum was acquired and registered as an individual identity with sample number and data information, such as product information, temperature, relative humidity (%) and instrument serial number. The reflection spectrum from 400 to 2500 nm was measured at 2 nm intervals with an average of 32 scans. Spectral preprocessing techniques were trialed to best remove irrelevant information that could not be handled properly by the regression techniques (APPENDIX C).

NIR instrument diagnostics were assessed 30 minutes before scanning each day. The instrument had to pass these initial checks before scanning was allowed. In addition, a routine sealed check sample (nylon) was scanned each day after diagnostics and every 30 minutes during the scanning process to ensure consistent instrument performance. The instrument lab environment was monitored and controlled utilizing a dehumidifier and air conditioner. The samples were retained and analyzed in a room with 40-43 % relative humidity and temperature of 19-21 ^{o}C.

All of the sample spectrums were performed in duplicate, repacked between analyses, and reviewed to ensure consistent spectral conformance. The averaged spectra were used in the calibration model development. The sample spectra were also predicted by Cargill Inc. NIR calibrations to ensure the meals met the expected nutritional profile (i.e. moisture, protein) before use in future modeling. The spectra scans were analyzed within 72 hours of the GLS HPLC analysis.

HPLC analysis

A 50 g ground split sample was analyzed in triplicate for total GLS according to ISO method 9167-1 (ISO, 1992). A Waters 600 HPLC instrument (Milford, MA) equipped with a Waters model 486 UV tunable absorbance detector (Milford, MA) at a wavelength of 229 nm was used to analyze the meal samples for total GLS. GLS extraction was completed with 70% hot methanol. Sample purification was conducted with an ion exchange column as described in PAPER 1. Enzymatic desulfation was then achieved with purified sulfatase to form neutral desulfoglucosinolates. The desulfoglucosinolates were separated using a C18 column (CAPCELL PAK C18 Type: C18 AG 120 A; 4.6 mm × 150 mm, 5 um) with a flow rate of 0.5 ml/min at 30 °C. Elution of desulfoglucosinolates from HPLC was performed by a gradient system of water (A) and acetonitrile/water (25:75, v/v, B). The total running time was 45 min with a gradient as follows: 100 % A and 0 % B for 5 min, then in 35 min to 0 % A and 100 % B and in 5 min back to 100 % A and 0 % B. Individual GLS were identified in comparison with the retention time of the standard (ISO, 1992). Total GLS were determined by adding all of the individual GLS and were expressed as μmol/g of dry matter.

Development of the NIR equation

The NIR spectrum was modeled against the total GLS laboratory analysis with the computer software WinISI v. IV (Foss-NIRSystems, Inc., Hilleroed, Denmark). A 3D plot was developed to evaluate the discrimination efficiency of the NIR analysis. Spectral data were standardized by Standard Normal Variate (SNV) and pre-treated using a derivative transform (SNV+DT) calculation (Alciaturi et al., 1998). In order to define the calibration and external validation sample population, the samples were listed from lowest to highest concentration according to GLS concentration and divided into two subsets with a ratio of 3:1 (calibration:validation). The subsets were then reviewed to ensure similar sample representation and statistics (i.e. mean, range, standard deviation).

For the calibration development, the raw data, first derivative and second derivative math treatments were tested for the entire *Brassica* meal dataset and for each meal group (*Brassica napus*, *Brassica napus-canola only*, *Brassica juncea*). To correlate the spectral information and the total GLS, modified partial least squares (MPLS) was used as a regression method. The software determined the optimum number of MPLS prediction factors by cross-validation with the leave-one-sample-out method. Calibration models were compared using the coefficient of determination (R^2) between the NIR and the reference values and the standard error of calibration (SEC). The calibration equation was then validated with an external validation set. The R^2, RPD and the standard error of prediction (SEP) were used as criteria for evaluating the predication capability of the calibration (Williams and Sobering, 1993).

Modified partial least square regression loadings

An MPLS loading plot for the first factor was calculated from the NIR MPLS regression, performed on the second derivative transformation of the raw optical data (2, 5, 5, 2) for total

GLS. MPLS regression constructs its factors capturing as much of the variation in the spectral data as possible by using the reference values actively during the decomposition of the spectral data. The loading plots show the regression coefficients of each wavelength to the parameter being calibrated for each factor of the equation. Wavelengths represented in the loading plots as participating more highly in the development of each factor are those which show more variation and which are better correlated to the analyte in the calibration set.

Average spectrum of *Brassica* meals

The second derivative average NIR spectrum of *Brassica* meals used in this work was obtained to identify and relate different absorption bands of the spectrum to specific absorbers influencing the MPLS loadings. In the first step, the original absorbance values of all samples at each wavelength (raw optical data from 400 to 2500 nm, every 2 nm) were averaged. The resulting average spectrum was standardized by using the algorithms SNV+DT. In a second step, the standardized spectrum was transformed into its second derivative (2, 5, 5, 2). The second order derivative transformation of the original spectrum results in a spectral pattern display of absorption peaks pointing downward.

Results and Discussion

Reference analysis

The meal collected from different seed varieties, growing seasons, and processing techniques had protein and moisture typical of RSM (Spragg and Mailer, 2007; Newkirk, 2009) (Table 12). The GLS range, mean and standard deviation varied widely among various RSM species and calibration/validation sets (Table 13, Figure 15). The HPLC GLS mean values differed among varieties where *B. juncea* (92.88 µmol/g) had the greatest mean GLS content while *B. napus* meal (40.60 µmol/g) the lowest. The *Brassica napus* canola samples had the

lowest GLS content, including standard deviation and range, which was expected as canola seeds are specifically bred to have low GLS concentration (<30 µmol/g). The total GLS concentration, obtained via HPLC, of the *Brassica* meal samples varied between 1.10-147.86 µmol/g. This range in total GLS was wider compared to previous research on intact whole rapeseeds, which ranged between 15.8-97.9 µmol/g (Petisco et al., 2010). This larger range was attributed to the elevated GLS concentration in the *B. juncea* samples. In addition, samples with high GLS were specifically targeted for this study during sample collection to improve the range used in the NIR calibration.

Table 12. Nutrient summary for NIR sample set.

Meal Type	Nutrient (%)	N[a]	Range	Mean	SD[b]
Brassica meal (all data)	Protein	186	32.43 - 42.31	37.25	1.47
	Moisture	186	7.41 – 13.78	11.51	1.26

[a] Number of samples
[b] Standard deviation

Table 13. Total glucosinolates (µmol/g) obtained from HPLC analysis of *Brassica napus* and *Brassica juncea.*

Datasets	N[a]	Range	Mean	SD[b]
Brassica napus (excluding canola)	100	3.47 - 138.56	40.60	39.67
Brassica napus (canola only)	63	1.01 - 25.27	9.52	5.15
Brassica juncea	23	8.01 - 147.86	92.88	45.08
Brassica meal (all data)	186	1.01 - 147.86	36.54	41.77
Global Calibration Dataset	140	1.01 - 147.86	36.58	42.01
Global Validation Dataset	46	1.59 - 142.55	36.40	41.51

[a] Number of samples
[b] Standard deviation of the reference data

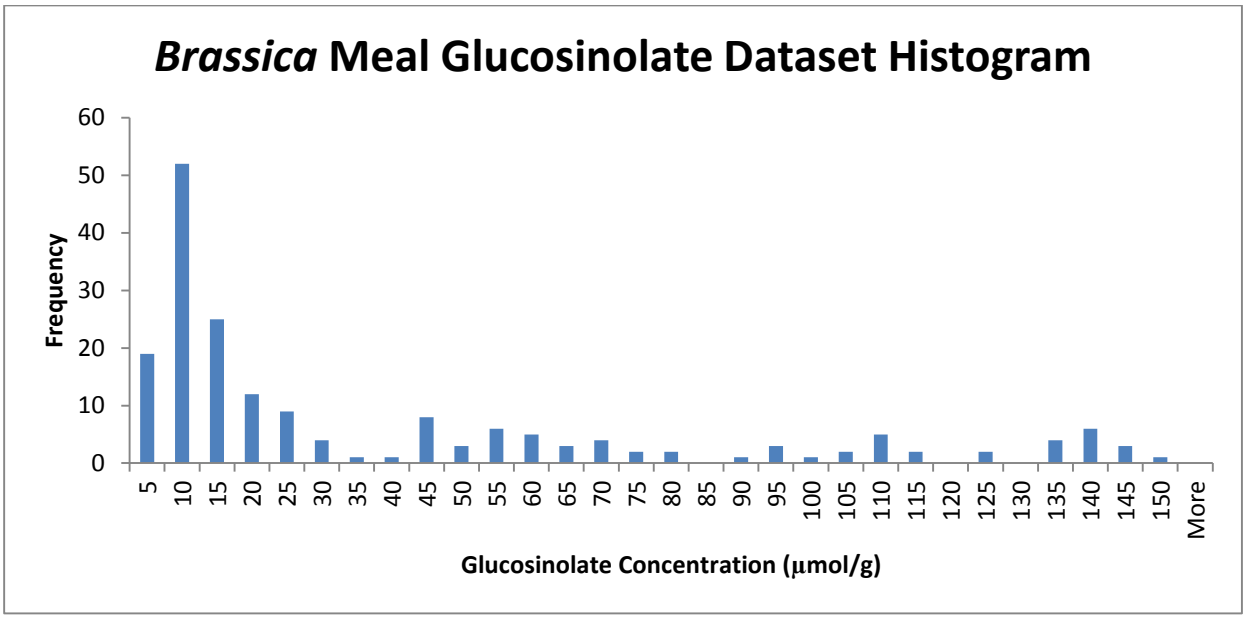

Figure 15. *Brassica* meal glucosinolate dataset histogram.

The diversity of the scanned meal samples demonstrates the diversity of the GLS in the samples collected for this study (Figure 16). The spectras were collected over the range of 400 to 2500 nm. There was an obvious spread along the Y-axis that was related to changes in reflectance (i.e. Log 1/R) for the different samples; however, the spectral pattern across the range of wavelengths (X-axis) appeared to be very similar for all samples. The visible region absorbance values were higher for *B. napus* compared to *B. juncea.* This was likely caused by the apparent color difference between the dark gray meals of the *B. napus* compared to the lighter colored *B. juncea* meal samples.

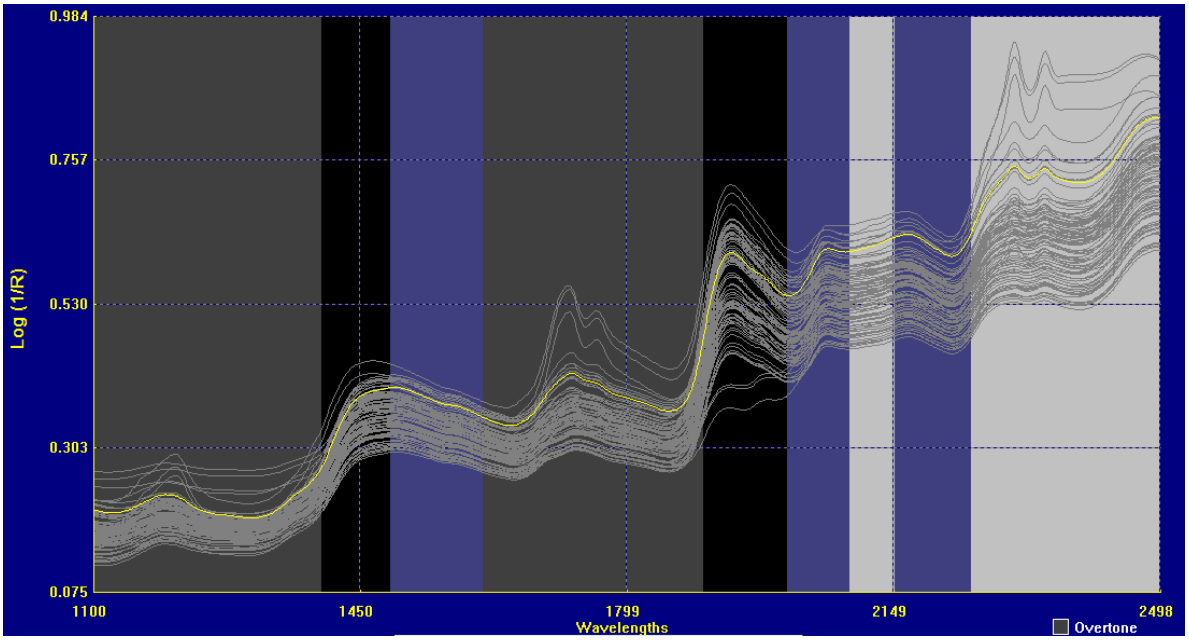

Figure 16. Near Infrared spectra for *Brassica* meal samples.

The main absorption bands in the near-infrared region are ascribable to the contribution of water (1460; 1940 nm), aliphatic chains of fat components (1210; 1715-1750; 2340 nm) and peptide bonds and amide groups of protein (2052; 2300 nm). Bands for moisture, fat and protein were respectively identified as 2nd overtone of –OH stretch, 3rd overtone –CH stretch, and 2nd and 3rd overtones of –NH stretch (Murray and Williams, 1987; Osborne et al., 1993).

MPLS loadings

In an attempt to explain the mechanism used by MPLS for modeling the low concentrations of GLS found in the samples, the spectrum of the *Brassica* meal calibration set was obtained and compared to the first MPLS loading plot of the equation for total GLS. Figure 17 shows the 2, 5, 5, 2 mean spectrum of the *Brassica* meal samples used in the calibration set (N=140) for the 1st MPLS loading plot for factor 1. The main absorption bands were displayed at 1922 nm, which was attributed to O-H stretch plus O-H deformation of water; 2056 nm related

to N-H stretch of amides; 2272 nm assigned to O-H plus C-C stretch groups (Osborne et al. 1993), and at 2310 and 2348 nm related to C-H stretching and combination bands of methylene groups (Murray & Williams, 1987).

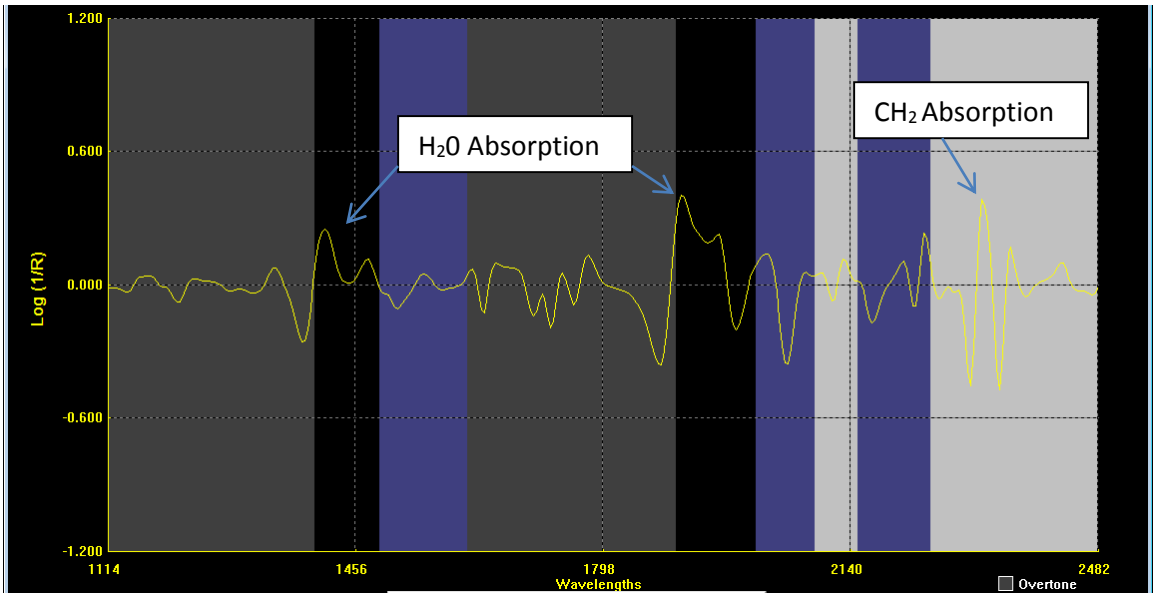

Figure 17. MPLS loading plots for factor 1 of the 2, 5, 5, 2 (SNV+DT) transformation for total glucosinolates.

The first MPLS term of the equation for total GLS was highly influenced by water absorptions at 1412 and 1908 nm, and also by absorption at 2300 nm, which is in the spectral region assigned to the C-H combinations of CH_2 groups (Murray & Williams, 1987). The major *Brassica napus* GLS found in the calibration set with the primary HPLC method were progoitrin, gluconapin, glucobrassicanapin, napoleiferin, glucobrassicin, and neoglucobrassicin (Table 14), which is consistent with a previous study (Bell, 1984). Additional MPLS loadings were influenced by wavelengths related to water (1924 nm), amides (2052 nm) and C-H combinations.

Table 14. Major glucosinolates found in *B. napus* and *B. campestris* rapeseed meals (Bell, 1984).

Glucosinolate	Semi-systemic name	R
Progoitrin	2-OH-3-butenyl-	CH_2=CH-CHOH-CH_3
Gluconapin	3-butenyl-	CH_2=CH(CH_2)$_2$
Glucobrassicanapin	4-pentenyl-	CH_2=CH(CH_2)$_3$
Napoleiferin	2-OH-4-pentenyl-	CH_2=CH-CH_2-CH-CH_2-OH
Glucobrassicin	3-indolyl-methyl-	$C_{16}H_{20}N_2O_9S_2$
Neoglucobrassicin	1-methoxy-3-indoly-methyl	$C_{17}H_{21}N_2O_{10}S_2^-$

The 3D representation of the principle components for all meal samples and harvest years indicates that majority of samples appear to be similar as they form a consistent cloud in the center of the plot (Figure 18). A few sample outliers were identified and investigated to ensure correct labeling. After review, four samples were determined to be mislabeled and were removed from the population statistics and NIR model. These samples were determined to be extruded RSM samples from China and were not included in the calibration sample set, reducing the sample size from 190 to 186.

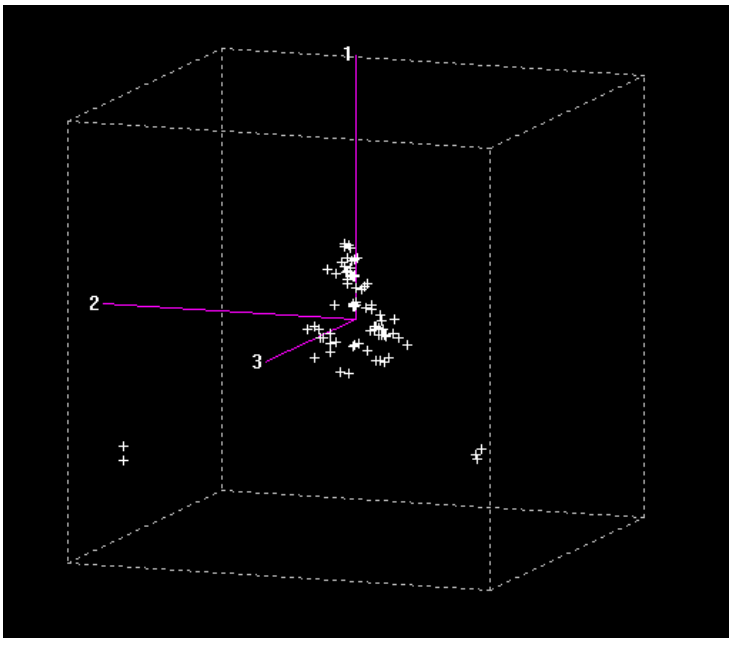

Figure 18. 3D principle component score plot for *Brassica* meal sample set.

NIR calibration

To test the prediction ability of the global calibration equation, an external validation set was used. The range of the external validation set was included within the values of the calibration data set, which is suitable for obtaining a successful validation (Shenk, 2001). The calibration statistics are dependent on the values obtained in the wet chemistry method. When the reference method is imprecise, the precision of predicting composition of unknown samples with NIR will also be imprecise. This will be reflected with a greater NIR SEP and lower R^2. The size of the SEP generally varies directly with the SD of the reference method. Given the SD of our assay for RSM was 2.25 µmol/g, our SEP should be 2 to 3 times the SEP of our validation set (6.05) and the R^2 should be above 0.90 (0.95) for a moderately robust calibration (Shenk, 2001), which was reflected in our model (Table 15). The individual species types (e.g. *Brassica napus, Canola, Brassica juncea*) were also modeled separately (Table 16).

Table 15. Calibration and external cross-validation statistics for the total glucosinolates (µmol/g) equation (N=140).

		Global Calibration		Global Validation		
Component	No. factors	R^{2a}	SEC[b]	R^{2c}	SEP[d]	RPD[e]
NIR - Total GLS	7	0.96	6.05	0.95	6.05	6.32

[a] Coefficient of multiple determination
[b] Standard error of calibration
[c] Coefficient of determination
[d] Standard error of prediction
[e] Relative predictive determinant

Table 16. Calibration and external cross-validation statistics for total glucosinolates (μmol/g) for individual NIR calibrations for the *Brassica* meal sub groups.

			Calibration	
Component	No. factors	No. samples	R^{2a}	SEC[b]
Brassica napus (excluding canola)	7	100	0.86	6.15
Brassica napus (canola only)	7	63	0.85	5.65
Brassica juncea	7	23	0.83	7.59

[a] Coefficient of multiple determination
[b] Standard error of calibration

The calibration model was developed by trialing different spectral pre-treatments (APPENDIX C). The best statistical results were obtained with the first derivative of the spectra. The relationship between chemical data and the NIR predicted values obtained from the global files of calibration and external validation for total GLS content supports the high correlation in the data (Figures 19, 20). The NIR software calculates the calibration and validation statistics. For total GLS content, the model showed correlation between NIRS and HPLC data with a R^2 of 0.96 and SEC of 6.05 (Table 15). Because there was no noticeable bias in the validation, the SEC will be discussed further with no bias reference. The SEC defines how well the NIRS prediction model predicts the reference value (calibration set) that was used to build the calibration. A low SEC is desired. As expected, the global calibration, which included all sample types, resulted in a better R^2 compared to models created for the individual species. This is likely a result of improved range in the global calibration for total GLS. The global calibration had a better SEC compared to the *Brassica napus* (excluding canola) (6.15) calibration and the *Brassica juncea* (7.59) only models but the canola only calibration had the lowest SEC (5.65). This was expected as the canola calibration GLS range was limited (1.01 - 25.27 μmol/g). The global calibration

had improved range, R^2, and is a more robust model for industry application compared to creating individual models for each species type.

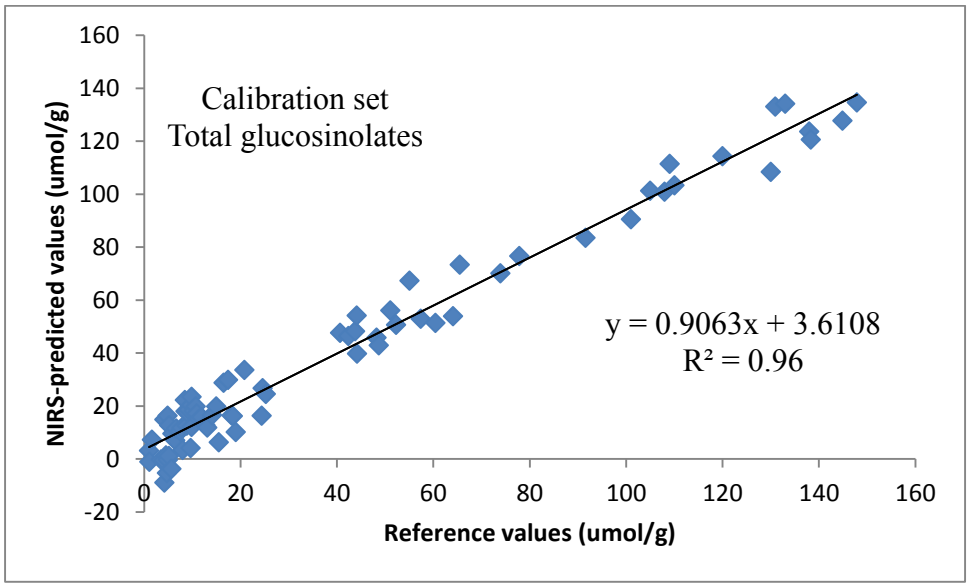

Figure 19. Scatter plots of reference and NIRS-predicted values obtained from the glucosinolate calibration set.

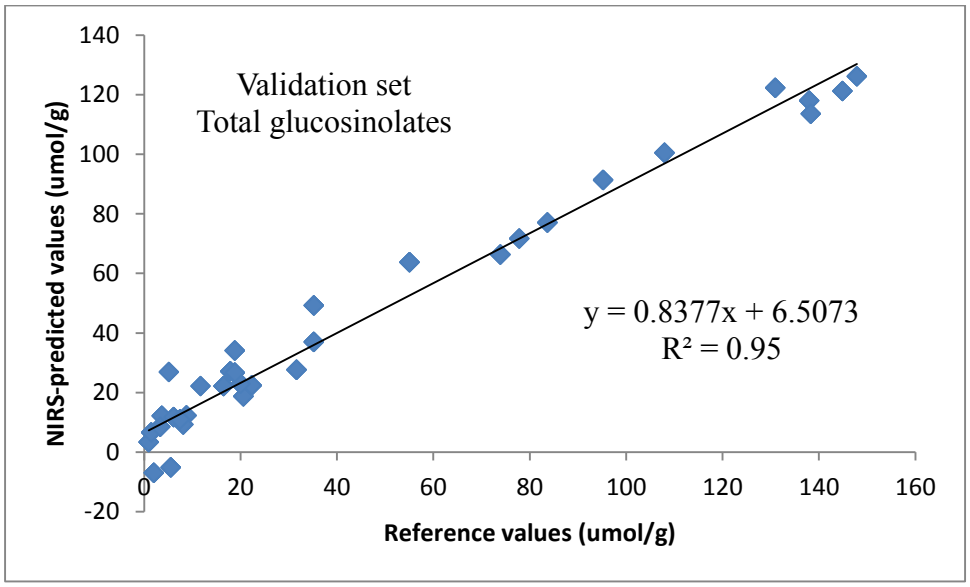

Figure 20. Scatter plots of reference and NIRS-predicted values obtained from the glucosinolate external validation set.

The external validation had an R^2 of 0.95, SEP of 6.05, and RDP of 6.32. The SEP defines how well the NIRS model predicts values for a validation set. A low SEP value is desired and in this study the validation appeared to perform similar to the calibration (SEC of 6.05 and SEP of 6.05). A high RPD value is desired and since our RPD was above 5 (6.32), our model is considered adequate for quantifying the desired analyte (total GLS) (Saeys et al., 2005). This suggests that a *Brassica* meal calibration can be improved when adding multiple *Brassica* species to extend the range and robustness of the calibration. This furthermore avoids the need to use specific calibration equations for single meal species.

There is a lack of NIR research on *Brassica* meals; therefore, the GLS calibration performance was compared to research conducted on whole *Brassica* seeds. The high values of R^2 with the global calibration model conducted in this study were in accordance with the results achieved by Velasco and Becker (1998). They reported R^2 of 0.99 and SEC of 4.1 μmol/g using 270 samples from several *Brassica* species within a range of 6 to 193 μmol/g. Other researchers modeling total GLS in *Brassica* seeds demonstrated similar results (R^2 values of 0.90-0.97 and SEC values of 5.01-5.80 μmol/g) (Hom et al., 2007; Evans and Bilsborrow, 1989). Our calibration also appeared to be satisfactory in comparison to calibration results reported by Daun et al. (1994) with Canola seeds (9.7-30.03 μmol/g). The model statistics differences between the studies can be attributed to multiple factors including the use of different NIR-software, reference methods and/or samples varieties (Hom et al., 2007; Salgo et al., 1992). But probably the main reason our GLS calibration SEC (6.05) was slightly higher compared to whole seed studies was attributed to sample presentation to the NIR. Although there are differences between seed varieties and region, additional meal processing differences were introduced into our model, which may have added more variety and error to the calibration.

Conclusion

The results obtained in this work demonstrated the adequate performance of NIRS technology in the quantification of GLS in *Brassica* meal samples after grinding. The global calibration equation was robust due to the variability of the sample set which included several varieties, harvests and processing techniques. In addition, the prediction accuracy of the unknown global validation set was comparable to studies conducted on whole seeds and resulted in a RPD of 6.32, supporting the hypothesis that a quantitative NIR model could be developed. The analysis speed and non-destructive sample property of this technique make it well adapted for quality control in oilseed crush facilities and feed manufacturing mills.

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PAPER 3. ALTERNATIVE *BRASSICA* MEAL PROCESSING METHODS EFFECTS ON GLUCOSINOLATE MODELING BY NEAR-INFRARED SPECTROSCOPY

Abstract

NIR has proven capable of predicting GLS quantitatively in *Brassica* meals. NIR modeling using unground *Brassica* meal samples and dried samples were analyzed in this study and compared to ground and "as is" moisture samples. NIR spectra data from 400 to 2500 nm at 2 nm intervals were recorded on unground *Brassica* meal samples (186) from various regions and growing seasons. A global calibration using the *Brassica* meal database was developed with a modified partial least squares regression (MPLS) analysis of conventional laboratory analysis. The calibration preprocessing included the first derivative and standard normal variate data preprocessing. The unground calibration was compared to a ground NIR model utilizing the same sample set and mathematical treatments. The unground NIRS calibration for total GLS resulted in a coefficient of determination (R^2), standard error of the calibration (SEC), and a relative predictive determinant (RPD) of 0.93 and 7.65, and 5.88 respectively.

A sample set (20) with known GLS concentration (by HPLC) was split and one subset was analyzed via NIR "as is" and the other subset was analyzed by NIR after drying for 16 hours at 60 ^{o}C in a vacuum oven. The dried *Brassica* meal sample set had a slightly better residual (HPLC - NIR) standard deviation (4.57) and average residual (-0.74), compared to the "as is" moisture sample set standard deviation (5.00) and average residual (-1.26).

Introduction

GLS are a group of *B*-D-thioglucosides that are mainly found in the family *Brassicaceae*. Many plants of this family are used in agriculture and nutrition, including rapeseeds. More than 120 different GLS are known (Kjaer and Skrydstrup, 1987). GLS are relatively nontoxic (Bell,

1984), but they gain importance from the fact that the products of myrosinase induced degradation adversely affect animal growth, reproduction, and performance as well as intake and palatability. The degradation products also cause goiter and abnormalities in internal organs of animals (Mawson et al., 1993).

Several methods have been developed to detect GLS and their hydrolysis products. Methods that indicate the total amount of GLS are often based on the measurement of released glucose or bisulfate ions by enzymatic assays (Thies, 1985). The method of choice is the determination of individual GLS by HPLC of the desulfoglucosinolates, which became established as the official reference method (high-performance liquid chromatography of desulfoglucosinolates) of the International Organization for Standardization (ISO, 1992). Although, chemical analysis of *Brassica* crops for determining the GLS content is expensive and time-consuming. The high cost and labor input required to obtain the total GLS content in the meal samples by the standard methods of analysis are serious handicaps to the analysis of large sets of samples. The use of fast analytical techniques such as NIR results in many advantages because analysis can be made with a considerable saving of time, at a low cost, and without using hazardous chemicals. In addition, samples can be analyzed in their natural form without destruction, which is very useful in the case of scarce and valuable samples.

NIR has proven capable of predicting GLS quantitatively in *Brassica* meals (PAPER 2). Sample preparation methods will be reviewed in this study to determine effects on calibration prediction accuracy. It is expected that an NIR calibration built with unground ("as is") *Brassica* meal samples will have an increased SEC, and a decreased R^2 and RPD compared to an NIR model built with ground meal samples. It is also hypothesized that drying meal samples down to reduce moisture will improve the NIR prediction accuracy of total GLS.

Materials and Methods

Sample preparation

This study consisted of evaluating the same 186 *Brassica* meal samples utilized in PAPER 2. The sample set represented different geographical regions (North America, Europe, and Asia) and multiple crop harvests between 2012-2013 (APPENDIX B). The samples were mixed thoroughly by hand and split into three 50 g sub-groups. Two of the sample sub groups were ground to pass through a 1 mm screen using a cyclonic mill (Retsch GmbH & Company, Hann, Germany). The third sample set was retained in its original state and analyzed unground on the NIRSystem 6500 for this study.

In addition, 20 of the 46 ground global validation set samples from Paper 2 were dried in a vacuum oven at 60 ^{o}C for 16 hours. The samples were then stored in a desiccator for 30 minutes to control the sample humidity and to bring the samples to room temperature (20-23 ^{o}C). These samples were then analyzed for total GLS with a FOSS NIRSystem 6500 with the same Global MPLS NIR calibration created in PAPER 2.

NIR spectra acquisition

The 186 unground samples were analyzed by a NIRSystems 6500 spectrophotometer (Foss-NIRSystems, Inc., Hilleroed, Denmark) equipped with a transport module, in the reflectance mode. Both visible and NIR spectra were collected utilizing lead sulfide (1100 - 2500 nm) and silicon (400 - 1100 nm) detectors. A total of 30 g of unground sample was packed in the NIRS quarter cup made of quartz glass and the spectrum was acquired and registered as an individual identity with sample number and data information, such as product information, temperature, relative humidity (%) and instrument serial number. The reflection spectrum from 400 to 2500 nm was measured at 2 nm intervals with an average of 32 scans. The same spectral

preprocessing techniques were trialed as in PAPER 2 to best remove irrelevant information that could not be handled properly by the regression techniques (APPENDIX D).

NIR instrument diagnostics were assessed 30 minutes before scanning each day. The instrument had to pass these initial checks before scanning was allowed. In addition, a routine sealed check sample (nylon) was scanned each day after diagnostics and every 30 minutes during the scanning process to ensure consistent instrument performance. The instrument lab environment was monitored and controlled utilizing a dehumidifier and air conditioner. The samples were retained and analyzed in a room with 40-43 % relative humidity and temperature of 19-21 ºC. All of the sample spectrums were performed in duplicate, repacked between analyses, and reviewed to ensure consistent spectral conformance. The averaged spectra were used in the calibration model development.

NIR Modeling

The unground NIR spectrum was modeled against the total HPLC GLS laboratory analysis conducted in PAPER 2 with the computer software WinISI v. IV (Foss-NIRSystems, Inc., Hilleroed, Denmark). Spectral data were standardized by the Standard Normal Variate (SNV) calculation (Alciaturi et al., 1998). The unground calibration (n = 140) and validation (n = 46) was comprised of the same samples utilized in PAPER 2 and the calibration/validation statistics were compared.

Results and Discussion

Unground NIR calibration

The unground NIR calibration model was developed by trialing the same spectral pre-treatments used in PAPER 2 for the ground calibration with the same sample set (APPENDIX D) and wet chemistry reference data. The best statistical results were obtained with the first

derivative of the spectra, which was similar to the ground calibration. The ground calibration demonstrated a better relationship between chemical data and the NIR predicted values obtained from the global files of calibration and external validation for total GLS content compared to the unground model (Table 17). For total GLS content, the ground model from PAPER 2 demonstrated correlation between NIRS and HPLC with a R^2 of 0.96 and a SEC of 6.05, while the unground calibration R^2 was 0.93, with an SEC of 7.65. The lower SEC for the ground calibration suggests that this model predicts the reference value (calibration set) more accurately compared to the unground calibration. This was an expected outcome as the NIR is very sensitive to light scattering and sample reflection. A ground sample is more homogeneous and would provide a more consistent surface for modeling, reducing potential modeling errors due to sample presentation.

Table 17. Calibration and external cross-validation statistics for a ground and unground total glucosinolates (µmol/g) NIR equation (N=140) for *Brassica* meals.

		Global Calibration		**Global Validation**		
Component	**No. factors**	**R^{2a}**	**SEC[b]**	**R^{2c}**	**SEP[d]**	**RPD[e]**
GLS Ground	**7**	**0.96**	**6.05**	**0.95**	**6.05**	**6.32**
Total GLS Unground	**7**	**0.93**	**7.65**	**0.94**	**7.88**	**5.88**

[a] Coefficient of multiple determination
[b] Standard error of calibration
[c] Coefficient of determination
[d] Standard error of prediction
[e] Relative predictive determinant

The external validation statistics for the ground calibration had a R^2 of 0.95, SEP of 6.05, and RPD of 6.32 (Table 17). The validation statistics for the unground calibration was 0.93 for R^2, SEP of 7.65, and a RPD of 5.88. A low SEP value is desired and in this study the validation appeared to perform similar to the calibration for both the ground and unground calibrations. In addition, a high RPD value is preferred and since the RPD was above 5 for both models, they are

considered adequate for quantifying the desired analyte (total GLS) (Saeys et al., 2005). This conclusion is important for the unground calibration from an industry application perspective. Not having to grind samples in the field, while maintaining confidence that the NIR will still be able to predict a reliable output is important for cost considerations and analysis speed.

Sample drying comparison

The effect of sample moisture on NIR modeling accuracy was analyzed. Twenty ground NIR validation samples from PAPER 2, which were analyzed for total GLS (by HPLC), total GLS (by NIR), moisture, and protein, were dried for 16 hours at 60 ^{o}C (Table 18). The sample processing step resulted in a reduced sample set average, range and standard deviation, compared to the original sample set. The non-dried moisture sample set had an average of 12.88 %, a range of 7.41-12.88 %, and a standard deviation of 1.39 %. The dried sample set averaged was reduced to 7.15 %, with a range of 5.89 – 7.15 %, and a standard deviation of 0.43 % moisture.

Table 18. Sample set moisture (%) as is and after drying, protein (%) and total glucosinolate concentration (µmol/g) by HPLC.

Sample ID	Crop Year	Region	Type	Moi[a]	Moi[b]	Protein	GLS[c]
CANA1226	2012	NA	Canola	11.53	5.89	38.34	3.55
EURA1317	2013	EU	Rapeseed	11.32	6.28	36.55	4.71
EURA1215	2012	EU	Rapeseed	7.41	6.36	32.76	5.21
EURA1204	2012	EU	Rapeseed	11.73	5.92	37.37	5.89
CANA1207	2012	NA	Canola	11.66	6.39	37.63	7.49
ASMU1206	2012	Asia	Mustard Meal	11.16	5.99	37.91	8.01
CANA1214	2012	NA	Canola	12.18	6.96	35.18	8.84
CANA1218	2012	NA	Canola	11.12	6.43	38.84	9.32
CANA1222	2012	NA	Canola	11.18	7.15	37.43	10.22
EURA1214	2012	EU	Rapeseed	9.95	6.36	35.67	10.99
CANA1334	2013	NA	Canola	11.09	7.11	35.43	14.11
EURA1306	2013	EU	Rapeseed	12.65	6.35	36.91	18.16
EURA1224	2012	EU	Rapeseed	12.64	6.89	38.02	21.53
EURA1312	2013	EU	Rapeseed	12.88	7.12	38.85	25.99
ASRA1310	2013	Asia	Rapeseed	12.09	6.88	38.45	44.13
ASMU1207	2012	Asia	Mustard Meal	11.64	6.12	35.67	51.33
EURA1218	2012	EU	Rapeseed	10.99	6.89	36.69	56.91
EURA1225	2012	EU	Rapeseed	11.00	7.01	38.07	67.89
EURA1209	2012	EU	Rapeseed	12.62	6.01	42.31	133.66
ASMU1211	2012	Asia	Mustard Meal	8.15	6.36	38.39	142.55

[a] % moisture as is
[b] % moisture after drying
[c] total glucosinolate concentration (µmol/g) by HPLC

The total GLS residuals (HPLC vs. NIR) were compared between the dried and non-dried sample sets (Table 19). The HPLC data served as the reference value and the NIR prediction for total GLS for both dried and non-dried samples were subtracted from the reference value to determine the residual. The average residual for the non-dried samples was -1.26, with a standard deviation of 5.00. This was compared to the average residual for the dried sample of -0.74, with a standard deviation of 4.57. A lower residual average and standard deviation is desired, suggesting that the NIR calibration is more similar to the reference method. In this study, the

dried samples had a lower residual average (-0.74) and standard deviation (4.57) compared to the non-dried sample set (-1.26, 5.00).

Table 19. Comparison of total glucosinolate concentration (μmol/g) between HPLC and NIR.

Sample ID	Moi[a]	Moi[b]	GLS[c]	GLS[d]	Residual[e]	GLS[f]	Residual[g]
CANA1226	11.53	5.89	3.55	2.35	1.20	2.916	0.63
EURA1317	11.32	6.28	4.71	7.12	-2.41	7.48	-2.77
EURA1215	7.41	6.36	5.21	14.23	-9.02	8.42	-3.21
EURA1204	11.73	5.92	5.89	2.23	3.66	0.38	5.51
CANA1207	11.66	6.39	7.49	11.27	-3.78	11.54	-4.05
ASMU1206	11.16	5.99	8.01	9.65	-1.64	8.208	-0.19
CANA1214	12.18	6.96	8.84	6.65	2.19	6.99	1.85
CANA1218	11.12	6.43	9.32	5.88	3.44	14.35	-5.03
CANA1222	11.18	7.15	10.22	15.04	-4.82	16.23	-6.01
EURA1214	9.95	6.36	10.99	11.92	-0.93	11.33	-0.34
CANA1334	11.09	7.11	14.11	9.96	4.15	8.55	5.56
EURA1306	12.65	6.35	18.16	22.45	-4.29	20.53	-2.37
EURA1224	12.64	6.89	21.53	22.23	-0.70	23.55	-2.02
EURA1312	12.88	7.12	25.99	19.07	6.92	19.11	6.88
ASRA1310	12.09	6.88	44.13	51.81	-7.68	44.12	0.01
ASMU1207	11.64	6.12	51.33	56.08	-4.75	49.32	2.01
EURA1218	10.99	6.89	56.91	61.44	-4.53	62.33	-5.42
EURA1225	11.00	7.01	67.89	60.79	7.10	61.36	6.53
EURA1209	12.62	6.01	133.66	144.12	-10.46	152.11	-10.45
ASMU1211	8.15	6.36	142.55	141.45	1.10	144.53	-1.98

[a] % moisture as is
[b] % moisture after drying
[c] total glucosinolate concentration (umol/g) by HPLC
[d] total glucosinolate concentration (umol/g) by NIR with samples as is
[e] c-d
[f] total glucosinolate concentration (umol/g) by NIR with dried samples
[g] c-f

As demonstrated in PAPER 2 from the MPLS loading plots, the water molecule is being heavily absorbed and is a factor during the MPLS NIR for GLS in *Brassica* meals so reductions in this variable may be contributing to increased prediction accuracy for GLS. Adding dried samples to the calibration could improve the calibration prediction for these types of samples in the future. Although there was a slight improvement in the GLS prediction accuracy with dried

samples, this improvement would need to be weighed against the added time needed to dry the samples. In food/feed industry application a huge advantage for NIR is its ability to analyze samples rapidly. Exposing the sample to an extended drying period may help to improve sample stability before testing, but the small accuracy improvements may not be worth the extended analysis time.

Conclusion

As expected, when comparing a ground NIR calibration with an unground model for the same sample set the ground calibration was more accurate. Because the NIR is very sensitive to light scattering and sample reflection, and the ground sample is more homogeneous, this type of sample would provide a more consistent surface for modeling, reducing potential modeling errors due to sample presentation. Both calibrations (dried and "as is") produced a RPD value above 5, considering them both adequate for quantifying GLS. In addition, a small NIR prediction improvement for GLS was noticed for samples that were dried to a similar moisture percentage before analysis. This was expected as water is known to be heavily absorbed during NIR modeling so reducing the variability in this compound was thought to reduce prediction error. Although the unground NIR calibration accuracy was reduced compared to the ground model and the "as is" sample average prediction residual was higher compared to the dried samples, the differences were minimal. The value of increased accuracy will need to be weighed against the value of reduced analysis time for sample grinding and drying.

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OVERALL CONCLUSION

During this study it was concluded that GLS preservation within stored *Brassica* meals is possible for 18 months, and possibly longer, providing the seed meals are protected from exposure to moisture conditions that promote microbial growth (i.e. relative humidity above 80%) and potentially endogenous myrosinase hydrolysis (i.e. sample exposure to moisture). For future GLS studies, it is recommended to store meal samples in polyethylene bags at -20 ^{o}C or 22^{o}C to ensure sample integrity.

It was also concluded that NIRS modeling techniques are capable of correlating and quantifying total GLS in *Brassica* meal for both ground and unground samples. Modeling and prediction accuracy can be improved through samples processing (sample drying and grinding). NIRS is a viable, rapid method alternative to predict totals GLS in *Brassica* meals. The analysis speed and non-destructive sample property of this technique make it well adapted for quality control in oilseed crush facilities and feed manufacturing mills.

FUTURE DIRECTION

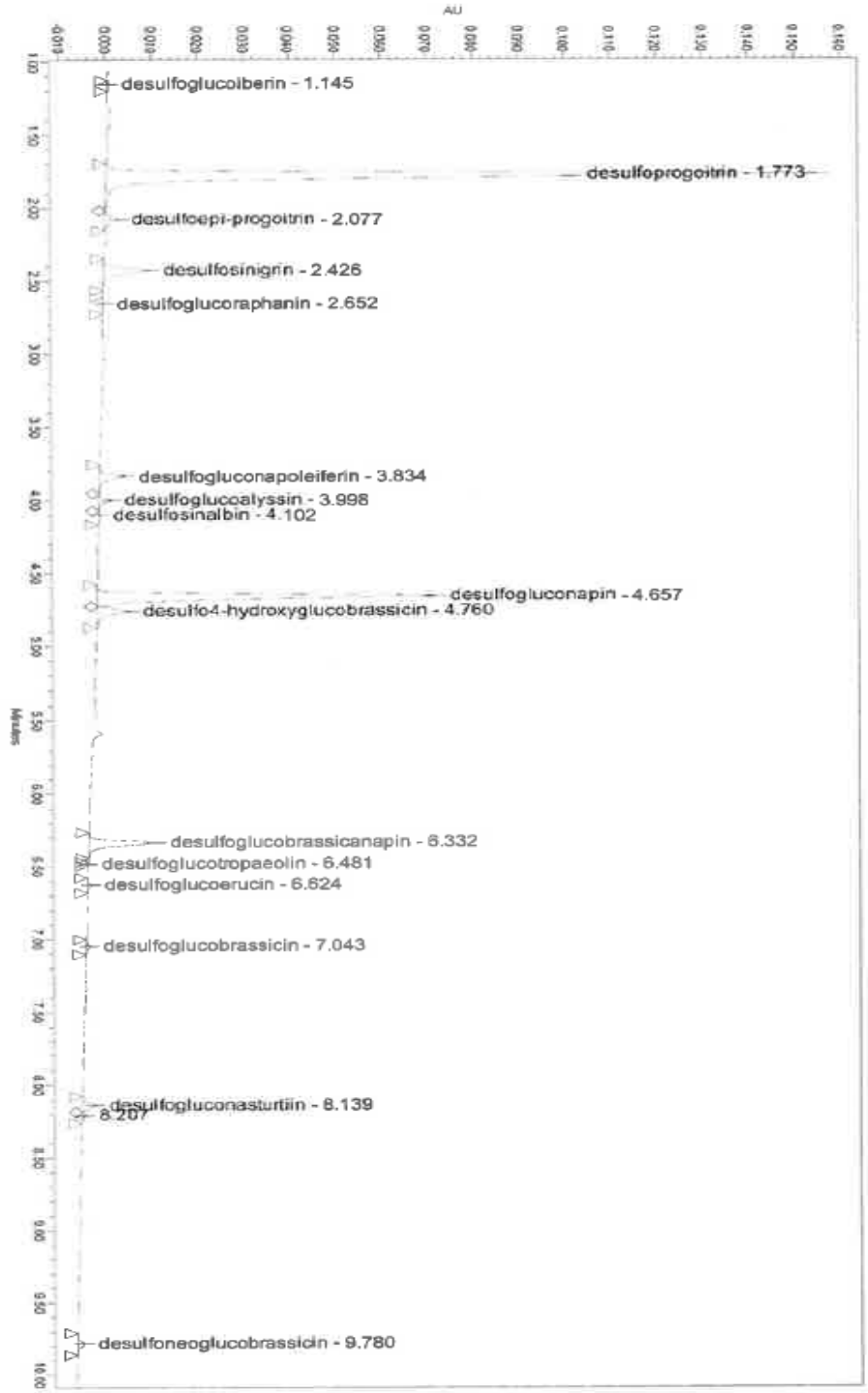
There were a few notable limitations for PAPER 1 that should be addressed in future studies. The humidity at certain storage temperatures appeared to promote microbial growth. The humidity was not well documented and controlled during the trial and should have been monitored more closely to better understand its effects. Packaging in nylon bags, due to their potential humidity barrier, should also be considered. In addition, this study did not quantify nor determine the type of visible microbial growth in the samples. Future studies should address these factors to better understand the effects of microbial growth on GLS during storage. Another limiting aspect of Paper 1 was the samples collected for this trial were selected based on their variety and total GLS concentration, not individual GLS profiles. Future studies should be designed to test the effects on individual GLS.

A limiting factor in PAPER 2 was the sample population. The samples were only collected over a few growing seasons, potentially limiting the model's robustness. Also, the NIR models lacked total GLS sample representation from 25-40 μmol/g. Now that a preliminary calibration has been built, future samples can be screened with this NIR model to determine if the samples will add value to the calibration. This capability should improve sample selection for calibration improvement. PAPER 3 only compared 20 dried validation samples to the ground calibration built in PAPER 2. Additional samples should be used to determine if drying meal prior to calibration development would significantly improve the calibration beyond the data obtained in PAPER 2.

The MPLS analysis is a viable option for modeling *Brassica* meal total GLS concentrations. Future studies should be administered to determine if different HPLC (i.e. Mass

Spectrometry) and NIR modeling techniques (ANN, local modeling) improve the models accuracy and repeatability. Also, future studies could be conducted to determine if quantitative NIR models can be created for individual GLS in *Brassica* meals. Addition studies should also concentrate on dried sample NIR calibrations to improve modeling accuracy. This would include building models and analyzing samples only comprised of dried samples to determine if other analytes are predicted better when sample moisture is less variable.

APPENDIX A. HPLC CHROMATOGRAM EXAMPLE FOR CANOLA MEAL



APPENDIX B. *BRASSICA* MEAL DATASET

Sample ID	Crop Year	Region	Type	Moisture (%)	Protein (%)	GLS (µmol/g)
ASMU1201	2012	Asia	*Brassica juncea*	9.11	38.71	142.37
ASMU1202	2012	Asia	*Brassica juncea*	9.13	39.10	64.09
ASMU1203	2012	Asia	*Brassica juncea*	8.51	35.85	138.44
ASMU1204	2012	Asia	*Brassica juncea*	9.16	36.40	138.29
ASMU1205	2012	Asia	*Brassica juncea*	8.19	38.07	144.85
ASMU1206	2012	Asia	*Brassica juncea*	11.16	37.91	8.01
ASMU1207	2012	Asia	*Brassica juncea*	11.64	35.67	51.33
ASMU1208	2012	Asia	*Brassica juncea*	7.99	38.84	147.86
ASMU1209	2012	Asia	*Brassica juncea*	10.84	37.97	67.89
ASMU1210	2012	Asia	*Brassica juncea*	12.78	38.15	135.32
ASMU1211	2012	Asia	*Brassica juncea*	8.15	38.39	142.55
ASMU1301	2013	Asia	*Brassica juncea*	11.05	38.84	42.35
ASMU1302	2013	Asia	*Brassica juncea*	10.82	36.88	90.33
ASMU1303	2013	Asia	*Brassica juncea*	11.41	37.12	48.20
ASMU1304	2013	Asia	*Brassica juncea*	12.21	40.38	132.33
ASMU1305	2013	Asia	*Brassica juncea*	9.11	36.97	110.21
ASMU1306	2013	Asia	*Brassica juncea*	12.95	37.81	112.54
ASMU1307	2013	Asia	*Brassica juncea*	13.41	38.14	91.60
ASMU1308	2013	Asia	*Brassica juncea*	9.89	37.60	108.00
ASMU1309	2013	Asia	*Brassica juncea*	12.93	38.59	106.33
ASMU1310	2013	Asia	*Brassica juncea*	10.29	37.70	65.51
ASMU1311	2013	Asia	*Brassica juncea*	11.86	37.76	35.44
ASMU1312	2013	Asia	*Brassica juncea*	12.13	35.35	12.33
ASRA1201	2012	Asia	*Brassica napus*	10.85	38.82	31.33
ASRA1202	2012	Asia	*Brassica napus*	9.47	37.22	60.44
ASRA1203	2012	Asia	*Brassica napus*	11.40	38.22	7.99
ASRA1204	2012	Asia	*Brassica napus*	12.95	37.70	5.43
ASRA1205	2012	Asia	*Brassica napus*	12.66	37.03	9.84
ASRA1206	2012	Asia	*Brassica napus*	11.30	36.01	11.00
ASRA1207	2012	Asia	*Brassica napus*	10.37	32.76	60.55
ASRA1208	2012	Asia	*Brassica napus*	13.11	38.88	101.33
ASRA1209	2012	Asia	*Brassica napus*	13.05	38.94	29.55
ASRA1210	2012	Asia	*Brassica napus*	9.35	36.72	77.84
ASRA1211	2012	Asia	*Brassica napus*	13.25	36.14	120.55
ASRA1301	2013	Asia	*Brassica napus*	11.45	38.89	8.60
ASRA1302	2013	Asia	*Brassica napus*	11.21	34.57	11.56
ASRA1303	2013	Asia	*Brassica napus*	11.04	36.70	51.06

Sample ID	Crop Year	Region	Type	Moisture (%)	Protein (%)	GLS (µmol/g)
ASRA1304	2013	Asia	*Brassica napus*	10.23	37.97	43.76
ASRA1305	2013	Asia	*Brassica napus*	12.20	34.36	11.90
ASRA1306	2013	Asia	*Brassica napus*	12.76	37.19	99.33
ASRA1307	2013	Asia	*Brassica napus*	9.55	37.37	74.33
ASRA1308	2013	Asia	*Brassica napus*	8.44	35.70	138.56
ASRA1309	2013	Asia	*Brassica napus*	9.74	37.60	138.29
ASRA1310	2013	Asia	*Brassica napus*	12.09	38.45	44.13
ASRA1311	2013	Asia	*Brassica napus*	11.12	36.50	44.20
ASRA1312	2013	Asia	*Brassica napus*	11.32	36.68	48.70
ASRA1313	2013	Asia	*Brassica napus*	11.66	36.80	130.78
ASRA1314	2013	Asia	*Brassica napus*	10.49	36.95	123.39
ASRA1315	2013	Asia	*Brassica napus*	9.52	38.94	78.33
ASRA1316	2013	Asia	*Brassica napus*	11.30	37.38	53.22
ASRA1317	2013	Asia	*Brassica napus*	12.51	34.92	44.20
ASRA1318	2013	Asia	*Brassica napus*	12.88	37.63	109.53
ASRA1319	2013	Asia	*Brassica napus*	11.67	38.68	130.94
CANA1201	2012	NA	*Canola*	12.95	38.94	8.90
CANA1202	2012	NA	*Canola*	9.15	37.55	1.01
CANA1203	2012	NA	*Canola*	11.76	35.52	12.69
CANA1204	2012	NA	*Canola*	9.40	38.26	1.50
CANA1205	2012	NA	*Canola*	9.93	38.71	1.59
CANA1206	2012	NA	*Canola*	11.04	36.67	9.84
CANA1207	2012	NA	*Canola*	11.66	37.63	7.49
CANA1208	2012	NA	*Canola*	13.50	37.31	15.51
CANA1209	2012	NA	*Canola*	11.95	36.77	16.35
CANA1210	2012	NA	*Canola*	10.53	38.26	16.50
CANA1211	2012	NA	*Canola*	11.69	37.89	8.45
CANA1212	2012	NA	*Canola*	10.85	36.66	8.55
CANA1213	2012	NA	*Canola*	13.05	39.01	8.45
CANA1214	2012	NA	*Canola*	12.18	35.18	8.84
CANA1215	2012	NA	*Canola*	11.20	35.03	14.50
CANA1216	2012	NA	*Canola*	11.84	38.45	10.30
CANA1217	2012	NA	*Canola*	12.51	37.76	9.29
CANA1218	2012	NA	*Canola*	11.12	38.84	9.32
CANA1219	2012	NA	*Canola*	12.41	35.22	15.51
CANA1220	2012	NA	*Canola*	10.93	37.38	14.98
CANA1221	2012	NA	*Canola*	12.09	35.74	8.94
CANA1222	2012	NA	*Canola*	11.18	37.43	10.22
CANA1223	2012	NA	*Canola*	11.58	38.00	10.30
CANA1224	2012	NA	*Canola*	11.43	38.35	14.29
CANA1225	2012	NA	*Canola*	11.64	38.72	10.84

Sample ID	Crop Year	Region	Type	Moisture (%)	Protein (%)	GLS (µmol/g)
CANA1226	2012	NA	*Canola*	11.53	38.34	3.55
CANA1227	2012	NA	*Canola*	11.67	36.64	5.67
CANA1301	2013	NA	*Canola*	12.63	34.24	4.08
CANA1302	2013	NA	*Canola*	13.25	39.39	5.60
CANA1303	2013	NA	*Canola*	11.84	37.54	5.88
CANA1304	2013	NA	*Canola*	11.22	35.11	13.10
CANA1305	2013	NA	*Canola*	12.99	34.84	17.40
CANA1306	2013	NA	*Canola*	9.40	38.68	1.10
CANA1307	2013	NA	*Canola*	12.53	35.80	4.88
CANA1308	2013	NA	*Canola*	10.79	35.79	4.92
CANA1309	2013	NA	*Canola*	12.63	38.47	7.88
CANA1310	2013	NA	*Canola*	12.89	38.21	9.25
CANA1311	2013	NA	*Canola*	9.78	39.85	1.74
CANA1312	2013	NA	*Canola*	11.60	36.40	1.80
CANA1313	2013	NA	*Canola*	11.66	37.97	5.67
CANA1314	2013	NA	*Canola*	12.76	38.94	6.43
CANA1315	2013	NA	*Canola*	12.81	37.49	10.70
CANA1316	2013	NA	*Canola*	13.05	36.12	8.94
CANA1317	2013	NA	*Canola*	11.72	36.97	7.49
CANA1318	2013	NA	*Canola*	12.15	36.64	4.66
CANA1319	2013	NA	*Canola*	12.44	37.88	10.92
CANA1320	2013	NA	*Canola*	11.86	39.10	8.55
CANA1321	2013	NA	*Canola*	9.89	36.68	5.14
CANA1322	2013	NA	*Canola*	11.54	38.30	5.11
CANA1323	2013	NA	*Canola*	12.10	37.82	8.35
CANA1324	2013	NA	*Canola*	10.93	36.40	14.90
CANA1325	2013	NA	*Canola*	10.84	34.24	15.51
CANA1326	2013	NA	*Canola*	10.49	38.07	5.67
CANA1327	2013	NA	*Canola*	11.80	38.02	7.76
CANA1328	2013	NA	*Canola*	13.23	38.82	9.00
CANA1329	2013	NA	*Canola*	10.90	38.61	10.57
CANA1330	2013	NA	*Canola*	13.19	37.76	9.25
CANA1331	2013	NA	*Canola*	12.44	37.35	9.84
CANA1332	2013	NA	*Canola*	11.00	35.15	12.55
CANA1333	2013	NA	*Canola*	11.76	36.17	16.99
CANA1334	2013	NA	*Canola*	11.09	35.43	14.11
CANA1336	2013	NA	*Canola*	12.92	36.66	25.07
EURA1201	2012	Europe	*Brassica napus*	12.47	35.31	4.20
EURA1202	2012	Europe	*Brassica napus*	11.62	36.49	3.47
EURA1203	2012	Europe	*Brassica napus*	11.73	38.45	8.33
EURA1204	2012	Europe	*Brassica napus*	11.73	37.37	5.89

Sample ID	Crop Year	Region	Type	Moisture (%)	Protein (%)	GLS (µmol/g)
EURA1205	2012	Europe	*Brassica napus*	12.51	37.22	5.20
EURA1206	2012	Europe	*Brassica napus*	11.61	38.30	56.32
EURA1207	2012	Europe	*Brassica napus*	12.27	37.00	22.56
EURA1208	2012	Europe	*Brassica napus*	13.16	38.19	108.54
EURA1209	2012	Europe	*Brassica napus*	12.62	42.31	133.66
EURA1210	2012	Europe	*Brassica napus*	10.96	38.88	6.44
EURA1211	2012	Europe	*Brassica napus*	13.00	38.09	7.96
EURA1212	2012	Europe	*Brassica napus*	12.47	35.17	4.56
EURA1213	2012	Europe	*Brassica napus*	10.49	36.75	18.43
EURA1214	2012	Europe	*Brassica napus*	9.95	35.67	10.99
EURA1215	2012	Europe	*Brassica napus*	7.41	32.76	5.21
EURA1216	2012	Europe	*Brassica napus*	11.58	34.52	48.00
EURA1217	2012	Europe	*Brassica napus*	12.79	36.69	52.28
EURA1218	2012	Europe	*Brassica napus*	10.99	36.69	56.91
EURA1219	2012	Europe	*Brassica napus*	11.25	36.72	6.30
EURA1220	2012	Europe	*Brassica napus*	11.44	35.83	12.70
EURA1221	2012	Europe	*Brassica napus*	11.58	38.66	9.62
EURA1222	2012	Europe	*Brassica napus*	9.11	39.10	5.23
EURA1223	2012	Europe	*Brassica napus*	12.88	32.43	5.21
EURA1224	2012	Europe	*Brassica napus*	12.64	38.02	21.53
EURA1225	2012	Europe	*Brassica napus*	11.00	38.07	67.89
EURA1226	2012	Europe	*Brassica napus*	13.73	39.08	105.00
EURA1227	2012	Europe	*Brassica napus*	11.54	37.11	19.03
EURA1228	2012	Europe	*Brassica napus*	12.14	38.21	40.56
EURA1229	2012	Europe	*Brassica napus*	10.45	36.88	6.44
EURA1230	2012	Europe	*Brassica napus*	13.24	37.54	7.96
EURA1231	2012	Europe	*Brassica napus*	11.06	35.11	12.89
EURA1232	2012	Europe	*Brassica napus*	11.55	37.47	19.33
EURA1233	2012	Europe	*Brassica napus*	10.52	37.19	6.44
EURA1234	2012	Europe	*Brassica napus*	12.21	34.52	10.89
EURA1235	2012	Europe	*Brassica napus*	11.27	35.43	55.03
EURA1237	2012	Europe	*Brassica napus*	12.51	38.78	109.00
EURA1238	2012	Europe	*Brassica napus*	11.73	36.67	88.90
EURA1239	2012	Europe	*Brassica napus*	11.49	38.08	52.69
EURA1240	2012	Europe	*Brassica napus*	11.77	38.72	44.35
EURA1301	2013	Europe	*Brassica napus*	11.41	37.23	9.81
EURA1302	2013	Europe	*Brassica napus*	12.23	36.30	3.78
EURA1303	2013	Europe	*Brassica napus*	13.04	36.37	22.67
EURA1304	2013	Europe	*Brassica napus*	11.29	36.36	4.19
EURA1305	2013	Europe	*Brassica napus*	12.36	37.60	55.45
EURA1306	2013	Europe	*Brassica napus*	12.65	36.91	18.16

Sample ID	Crop Year	Region	Type	Moisture (%)	Protein (%)	GLS (µmol/g)
EURA1307	2013	Europe	*Brassica napus*	11.75	35.93	4.89
EURA1308	2013	Europe	*Brassica napus*	9.59	36.64	73.91
EURA1309	2013	Europe	*Brassica napus*	9.72	37.70	136.89
EURA1310	2013	Europe	*Brassica napus*	11.98	38.78	6.66
EURA1311	2013	Europe	*Brassica napus*	10.90	36.48	20.80
EURA1312	2013	Europe	*Brassica napus*	12.88	38.85	25.99
EURA1313	2013	Europe	*Brassica napus*	9.68	39.39	67.33
EURA1314	2013	Europe	*Brassica napus*	11.52	35.85	4.05
EURA1315	2013	Europe	*Brassica napus*	11.32	36.28	9.83
EURA1316	2013	Europe	*Brassica napus*	12.09	38.29	20.88
EURA1317	2013	Europe	*Brassica napus*	11.32	36.55	4.71
EURA1318	2013	Europe	*Brassica napus*	13.14	38.39	91.60
EURA1319	2013	Europe	*Brassica napus*	13.07	37.89	24.39
EURA1320	2013	Europe	*Brassica napus*	11.16	37.81	7.76
EURA1321	2013	Europe	*Brassica napus*	13.78	35.13	19.55
EURA1322	2013	Europe	*Brassica napus*	12.32	39.01	24.60
EURA1323	2013	Europe	*Brassica napus*	11.11	38.19	6.63
EURA1324	2013	Europe	*Brassica napus*	12.15	37.97	9.67
EURA1325	2013	Europe	*Brassica napus*	12.20	36.68	57.36
EURA1326	2013	Europe	*Brassica napus*	11.45	37.76	40.63
EURA1327	2013	Europe	*Brassica napus*	12.02	37.59	11.40
EURA1328	2013	Europe	*Brassica napus*	12.02	34.57	55.00
EURA1329	2013	Europe	*Brassica napus*	10.36	38.59	20.03
EURA1330	2013	Europe	*Brassica napus*	10.36	34.93	12.34

APPENDIX C. TOTAL GLUCOSINOLATE PRETREATMENT NIR MODELING TRIAL (GROUND) CALIBRATION AND VALIDATION STATISTICS

preTreatment	SEC[a]	CR[2b]	SEP[c]	VR[2d]
none	22.86	0.72	23.53	0.71
SNV[e]	20.6	0.78	21.28	0.76
1st Der[f]	6.78	0.96	7.85	0.94
SNV + 1st Der	6.05	0.96	6.05	0.95
2nd Der[g]	9.52	0.95	7.95	0.93
SNV + 2nd Der	8.05	0.95	6.6	0.94
SNV& Detrending	16.79	0.85	20.04	0.79
SNV&D[h] + 1st der	6.05	0.96	6.79	0.95
SNV&D + 2nd der	8.13	0.95	7.55	0.94
SNV + 1st Der + Dwt[i]	6.78	0.97	7.12	0.95
SNV + 2nd Der + Dwt	8.74	0.94	8.56	0.93

[a] standard error of the calibration
[b] coefficient of determination of the calibration
[c] standard error of prediction of the validation
[d] coefficient of determination of the validation
[e] standard normal variate
[f] 1st derivative
[g] 2nd derivative
[h] standard normal variate and detrending
[i] Down-weighted

APPENDIX D. TOTAL GLUCOSINOLATE PRETREATMENT NIR MODELING TRIAL (UNGROUND) CALIBRATION AND VALIDATION STATISTICS

preTreatment	SEC[a]	CR[2b]	SEP[c]	VR[2d]
none	25.96	0.75	24.63	0.70
SNV[e]	24.6	0.73	21.55	0.73
1st Der[f]	8.78	0.90	8.99	0.86
SNV + 1st Der	7.65	0.93	7.88	0.94
2nd Der[g]	13.52	0.89	9.95	0.89
SNV + 2nd Der	9.25	0.91	8.62	0.93
SNV& Detrending	16.88	0.88	17.24	0.78
SNV&D[h] + 1st der	7.77	0.93	8.02	0.94
SNV&D + 2nd der	10.59	0.91	11.55	0.92
SNV + 1st Der + Dwt[i]	7.78	0.92	8.42	0.91
SNV + 2nd Der + Dwt	8.99	0.92	9.59	0.92

[a] standard error of the calibration
[b] coefficient of determination of the calibration
[c] standard error of prediction of the validation
[d] coefficient of determination of the validation
[e] standard normal variate
[f] 1st derivative
[g] 2nd derivative
[h] standard normal variate and detrending
[i] Down-weighted