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Particle size optimisation in development of near infrared microscopy methodology to build spectral libraries of animal feeds

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Near infrared spectroscopy coupled to a microscope (NIRM) is a technique where the infrared beam is focused on each particle (<50 µm) of a sample to collect NIR spectra. In this work, the experimental conditions (sample pre-treatment and instrumental) have been evaluated and compared for collecting spectral information in order to develop a strategy able to build large and robust spectral libraries to be transferred between NIRM instruments. To attempt this objective, we selected the most representative ingredients included in animal feeds: straw as low density forage, soybean as raw material and total mixed ration (TMR). Then, different mills, sieves and the number of spectra to be averaged were evaluated, selecting those that minimise noise and scanning time. The best results were obtained grinding through 1 mm sieve ingredients and TMR and collecting each particle spectra by scanning 70 individual spectra averaged as pre-treatment and instrumental conditions to obtain the most relevant information about each ingredient.

Keywords: near infrared (NIR) microscopy, particle size, spectral libraries, animal feeds

Introduction

Near infrared reflectance microscopy (NIRM) is an objective, sensitive and highly-selective technique for the safety and quality control of animal feeds. It combines the analytical advantages of microscopy and spectroscopy techniques.[1] The principle of NIRM is based on the collection of spectra from several particles contained in a sample. These spectra can be collected from extremely small particles (≤50 µm) using a Fourier transformer near infrared reflectance (FT-NIR) instrument attached to a microscope with a highly developed optical system and designed to focus on particles, increasing the efficiency of radiation transmission for microspectrum collection. On animal feeds, due to real sample heterogeneity, several hundreds of particles must be analysed, the result of which is a successive collection of hundreds of spectra. These spectra are the result of light absorption by organic molecules and a "unique" fingerprint that can identify each compound[2,3] and each single spectrum is the molecular near infrared signature of one particle. In this analysis by NIRM, the sample particles are spread on a sample holder and each particle is focused and selected using a viewing system to collect NIRM spectra. The final result of the sample NIRM analysis is a successive collection of spectra of different particles included in an ingredient or compound feed.

This method has the advantage of relying only on the chemical properties of samples and to be independent of human subjectivity; for this reason, ingredients used in the formulation of compound feeds have a large collection of spectra corresponding to their particles. Those ingredients included in the compound feed can be identified by comparing spectral information with reference spectral libraries prior to the

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development of a discriminant analysis with an appropriate chemometric tool.

Earlier studies using NIRM were developed in the pharmaceutical industry, demonstrating the useful role of this technique[2,4,5] because it offers the opportunity to explore what chemical species are present at micro-scale level in one sample, and it provides spatial information on their distribution within a sample. Nevertheless, NIRM was introduced into the agro-food industry in the 90's and first studies have demonstrated the high potential of this technique in the development of different methodologies for detection of animal meals in compound feeds.[6–11] In all these preliminary works, spectral libraries were constructed including thousands of spectra of single particles from animal, vegetal and mineral feed ingredients. The comparison of these reference spectral data with those from unknown animal feeds made it possible to identify the origin of each particle in the samples. It is important to point out that, in some cases, it was necessary to carry out a previous chemometric pretreatment of spectral data to remove the unwanted sources of variation of spectral information (humidity, temperature, etc.) to identify the vegetal or animal origin of each particle in the sample in order to minimise spectral interferences. The great advantages of this technique are that sample/particle recognition is not dependent on the expertise of the analyst and that it is possible to automate all procedures, increasing the number of samples analysed per unit of time compared with classical microscopy. Moreover, the spectral signature of each particle can be stored and used in further investigations.

It is well known that the development of a NIRM methodology to build up spectral libraries is not a simple task (tedious, time consuming ...). The most important step of the process is to establish the experimental basis and parameters to be optimised: particle size, distribution, surface texture and number of spectra to average. All those parameters should be appropriately selected for the generation of large and robust spectral libraries to be transferred between NIRM including all spectral information to characterise the ingredients included in animal feeds. All these experimental conditions represent the preliminary work for the development of identification methods for this methodology. Besides, the building of robust libraries able to be transferred between different instruments minimises the effort needed to establish an NIRM reference methodology for the prediction of ingredient composition. Moreover, it provides reference libraries containing more variability and spectral information available to further research.

The objective of present work was to evaluate, compare and establish the experimental conditions associated with sample preparation and instrumental NIRM data recorded (particle size, distribution, surfaced texture and the number of spectra to average) in order to build NIRM reference spectral libraries on animal feed with all relevant sample information, thus guaranteeing that the spectral information transferability between instruments located at different laboratories is similar.

Materials and methods

Samples

Different macro-ingredients representative in animal feeds and a complete ration were involved in this study: (1) straw as low density forage, (2) soybean as raw material and (3) total mixed ration (TMR) with maize silage as the complex mixture containing different ingredients. The particle size intervals were defined according to sample preparation methodology of the NIR analytical system on animal feed,[11,12] three different sieves were evaluated: 0.75; 1 and 2 mm using different and specific mills available in the lab, according to sample characteristics:

(a) Mill A (Pulverisette 25 Fritsch): this mill is suitable for coarse grinding of soft to medium-hard and fibrous dry animal feed. The milling cutter rotor has V-cutting edges and fixed knives made of tool steel. The sample exhauster has a cyclone separator. This mill can be used, for example, to crush animal feeds, straw, grains, farinaceous products, maize, malt, bones, etc. Due to the extremely wide range of possible applications, in this work we used this mill for grinding all the kinds of sample considered: straw, soybean and TMR.

(b) Mill B (Pulverisette 14 Fritsch): the operation method is based on a high-speed rotor with shaped ribs to crush the sample by impact factor and shearing. This mill is suitable when samples are difficult to grind, such as samples that include a lot of different ingredients with a wide variety of texture and hardness, such as TMR.

(c) Mill C (Cyclotec 1093 Foss Tecator): This mill is designed for rapid, uniform grinding of feed such as grains. In this study, this mill was used only for grinding soybean.

In order to obtain the sample characteristics and mill specifications detailed above, in this study we tried the mills and sieves detailed in Table 1.

NIRM analysis

In this work, an Auto Image Microscope connected to a Perkin-Elmer Spectrum One FT-NIR spectrometer in reflectance mode (1112–2500 nm) was used. Taking into consideration

Table 1. Samples, mills and sieves assayed to optimise particle size.

Sample	Mill	Sieve (mm)
Soybean	A	0.75
	A	1
	C	1
	A	2
Straw	A	0.75
	A	1
	A	2
Total mixed ration	A	0.75
	A	1
	B	1
	A	2

previous works in NIRM, the resolution employed was 8 cm^{-1}.[8,9] Spectra were obtained from the ratio between raw spectra and the background (measured using the spectralon plate) and the spectral information was stored as log (1/*R*). This instrument enables the collection of spectra from small surfaces (50 µm × 50 µm) and is equipped with an InGaAs detector, a camera and a viewing system to magnify the visible-light image and to localise and mark, one-to-one, the particles of interest to be analysed.

Previous works in our research group[9,11] have established the minimum number of particles to be analysed depending on sample uniformity. Following such results, in heterogeneous samples such as total mixed ration at least 500 particles must been scanned to guarantee that all necessary spectral information is recorded, whereas, for homogeneous samples such as soybean and straw, at least 200 particles should be scanned.

To attempt the objective planned for this work, it was necessary to optimise the strategies for random selection of particles to scan and the number of spectra to average. Considering all assays and treatments, first different alternatives were evaluated for random selection of particles in each sample using the microscope pointer: each scan point can be selected by the technician or in the middle of each individual visible view at the same time as focussing on the sample. In order to avoid the subjectivity of the analyst and to obtain spectral information able to represent the heterogeneity of each feed or ingredient, the automatic option was selected for further investigations, this being the most automated and random process. To minimise the noise in the base line the following conditions were assayed: 10, 20, 50, 70 and 100 scans to average by particle/spectra.

The software provided by PerkinElmer, Spectrum v. 5.01,[13] was used to collect and store spectra. WinISI II software, v.1.05,[14] was used for computer operation and spectral data collection analysis.

Mathematical and statistical analysis

In order to develop mathematical and statistical analysis, spectral data were exported from Spectrum software, v. 5.01, in ASCII format into WinISI II software, v.1.05.

In order to evaluate and select the best experimental sample conditions for NIRM analysis, we estimated the root mean squares of differences (*RMS*) between the spectra of one particle and the mean spectra of the all particles by feed/ingredient for each experimental condition. In order to reduce baseline offset arising from sample characteristics, scatter correction math treatment was performed on spectral data, applying standard normal variate and detrending (SNVD) of log (1/*R*) at each wavelength[15,16] and different derivative transformations using common mathematical treatments. Four mathematical treatments were used in the transformation of spectral data to reduce the root mean square between each particle and the mean population spectrum: 0,0,1,1; 1,10,5,1; 1,5,5,1 and 2,10,5,1, where the first number denotes the derivative order while the second number denotes the number of nanometres in the segment used to calculate the derivative (first or second derivative reduce spectral variance caused by particle size[17]). The third and fourth numbers denote the number of data points over which running average smoothing was conducted.

One of the most common chemometric tools widely used to detect acquisition spectral errors was applied, the CENTER algorithm[18] from WinISI II. The first step is to perform principal components analysis, which reduces the redundant spectral information to a few linearly independent variables, facilitating the calculation of spectral distances. After obtaining these new calculated Mahalanobis distance (*GH* statistic) for each sample by the standardised *H* distance from the mean, samples with large *GH* values (*GH* > 3) are considered as spectral outliers. Afterwards, the algorithm SELECT was applied[18] for choosing the most representative spectra/particles in each type of sample by elimination of particles with similar spectra from a spectra file by using Mahalanobis distance between all pairs of spectra, using 1.5 value as the NH cut-off.

Analysis of variance

Two different analyses of variance (ANOVA) were carried out to asses the relative importance of different sources of variation for the *RMS*, including type of mill, sieve and number of scans to be averaged as variables. The least square mean was compared with errors corresponding with the two-way factor interaction when optimising particle size (mill and sieve) and the one-way factor interaction for optimising the number of scans to average. Calculations were performed by using SAS software[19] with the GLM procedure to fit general linear models by relating one continuous dependent variable (*RMS* value) to several independent variables (mill, sieve and scans averaged).

Results and discussion

The lowest *RMS* results were obtained when using SNVD and applying 1,5,5,1 mathematical treatment. This treatment minimises the *RMS* value between the spectra of one particle and the mean spectra of all particles by feed/ingredient on each experimental condition. For example, *RMS* values obtained grinding TMR using Mill A through a 0.75 mm sieve were 70943, 5715, 4658 and 8035 working with raw spectra and 1,10,5,1; 1,5,5,1 and 2,10,5,1 with derivatised spectra, respectively. For straw and soybean, by employing the same experimental conditions, *RMS* values were 82384, 6019, 4361 and 8042; 106481, 12056, 10010 and 14925 for raw spectra and 1,10,5,1, 1,5,5,1 and 2,10,5,1 for derivatised spectra, respectively. The *RMS* values obtained with the math treatment selected (SNVD and 1,5,5,1) were the lowest for all the samples used.

Figure 1(a) shows the raw mean spectra obtained with different mills and Figure 1(b) the spectra registered after applying first derivative using the same experimental conditions

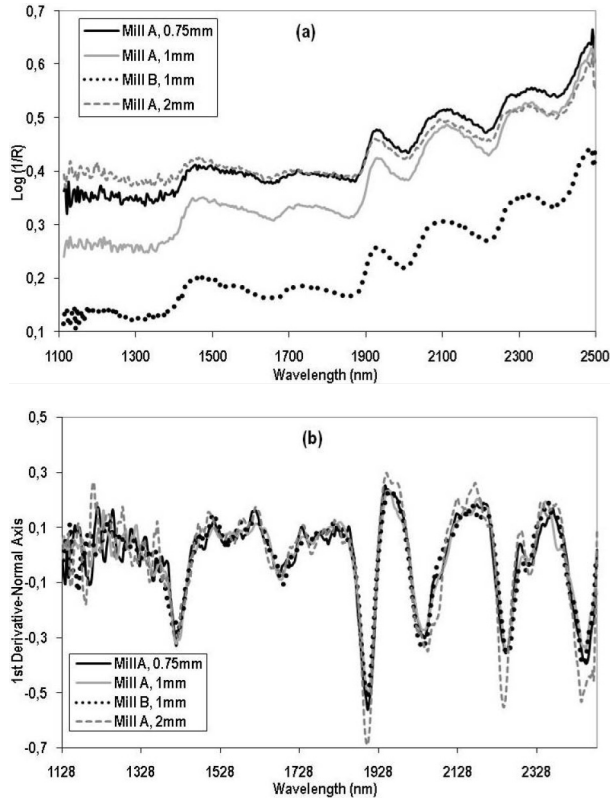

Figure 1. NIRM spectra of total mixed rations with 8 cm^{-1} resolution and 70 scans averaged using different mills and sieves: (a) raw spectra and (b) Derived spectra applying first derivative.

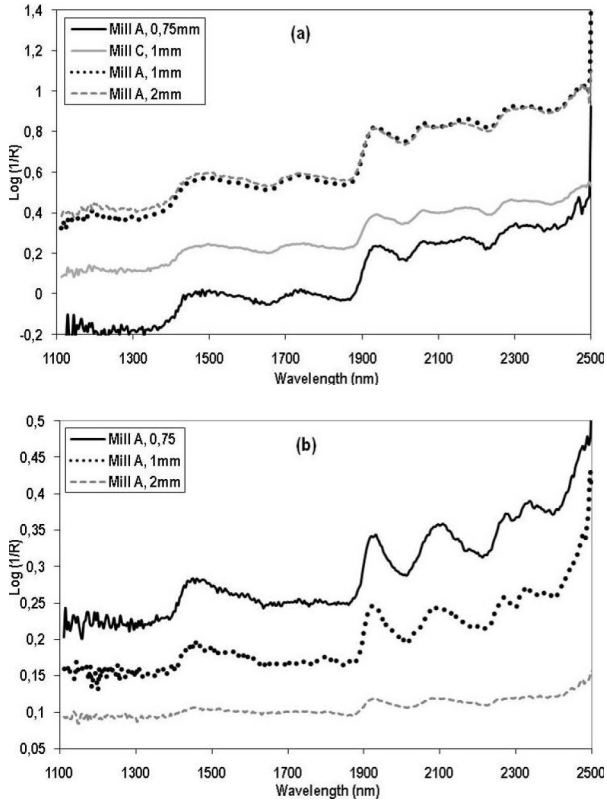

Figure 2. Raw NIRM spectra of macro-ingredients with 8 cm^{-1} resolution and 70 scans averaged using different mills and sieves: (a) soybean and (b) straw.

(mills and sieves) for the most complex matrix (TMR). In these figures, clear differences related to noise and robustness of spectral data are observed depending on the sample mill and sieve. Moreover, after applying the math pre-treatment, these differences are minimised [see Figures 1(a) and 1(b)]. Similar results are observed with forages and grains (see Figure 2).

Table 2. ANOVA results for optimising particle size and type of mill.

Sample	Mill	Sieve (mm)	RMS
Soybean	A	0.75	9692[b]
	A	1	9911[b]
	C	1	3620[a]
	A	2	13298[c]
Straw	A	0.75	4788[a]
	A	1	5299[a,b]
	A	2	5971[b]
Total mixed ration	A	0.75	5759[b]
	A	1	5081[b]
	B	1	2907[a]
	A	2	6597[b]

a–c: Different letters within column and within each kind of sample indicate statistically significant difference between the means ($p < 0.05$
RMS: root mean square of differences between spectral data

After developing the ANOVA test with different mills and particle sieves for all samples considered in this study (see Table 2). The lowest *RMS* value for soybean was obtained when ground with Mill C, which can only be used with a 1 mm sieve. Comparing the same particle size, the *RMS* differences between Mills A and C for soybean were 9911 and 3620, respectively. These values are due to the different technical characteristics of the mills (see material and methods). When using Mill C, a more uniform particle size distribution is obtained than when using Mill A, with a lower *RMS* and being statistically significant ($p < 0.05$). Similar results were obtained for the TMR samples. The most universal mill (A), can not be used for this sample. *RMS* results obtained when using Mill B with a 1 mm sieve were 2907 with significant differences for Mill A with all sieve sizes ($p < 0.05$). Probably, the sample exhauster system of Mill A, a strong air current to transport the sample, could produce a more fibrous consistency on ingredients with the same grinding difficulty. The TMR final particle size obtained with Mill B is more uniform and as referred to above, this effect is transduced to the lowest *RMS* values.

Straw can only be ground with Mill A because of technical characteristics. However we observed some differences in the particle size. The lowest *RMS* value in the straw samples was obtained at 0.75 mm (4788) without differences with a 1 mm

Table 3. ANOVA results for scans number to average/spectra on different ingredients of animal feeds.

Sample/scans	10	20	50	70	100
Soybean	9358[b]	8151[b]	4855[b]	3864[a]	3420[a]
Straw	7694[c]	5747[c]	4282[b]	4077[a,b]	3756[a]
Total mixed ration	6800[b]	5800[b]	4530[b]	4325[a]	3958[a]

a–c: Different letters within row indicate statistically significant difference between the means ($P < 0.001$). RMS: root mean square of differences between spectral data

sieve (5299) but statistical differences (5971, $p < 0.05$) were obtained with a 2 mm sieve.

Looking at these results, we can conclude that it is not possible to use the same mill for all kind of samples to build spectral libraries for heterogeneous samples. TMR need to be ground in a mill designed for samples with a wide variety of textures and can achieve fine grinding by impact and shearing. Homogeneous feed samples such as grains produced better spectra data (low *RMS* values) when using a mill capable of producing rapid and uniform grinding for which, in this work, we used Mill C.

The final results obtained in this study for soybean NIRM analysis were in agreement with the sample preparation recommended by the Canadian Grain Commission prior to NIR analysis.[20] Although the particle size is different for each kind of sample (1 mm for soybean, 0.75 mm for straw and 1 mm for TMR), it is possible to unify the grinding mill characteristics because there are no spectral data differences, on the basis of *RMS* values, when the straw is ground at 0.75 mm or 1 mm.

The ANOVA results obtained when studying interactions between the number of scans by particle (10, 20, 50, 70 and 100) and the type of sample, to evaluate the influence of the number of scans to be averaged when scanning each sample particle, are showed in the Table 3. As can be seen, in soybean and TMR the lowest *RMS* values were obtained with 100 scans (3420 and 3958, respectively) and without statistical differences between 70 scans (3864 and 4325) by particle. A number of scans per particle lower than 70 to average showed significant differences ($p < 0.001$) in both animal feeds. The best result in straw was 100 scans to average per particle, without significant differences between 100 and 70 scans. There were not differences between 70 and 50 scans to average per particle, although there were significant differences between 50 and 100 scans (4282 and 3756, respectively, $p < 0.001$).

Therefore, in order to unify the experimental conditions in this aspect we selected 70 as the number of scans to average by particle in all types of sample, because *RMS* results did not show significant differences between average 100 or 70 scans per particle. By reducing 30 measurements of scan data collection by particle, it is possible to save 38 minutes of analysis time by sample, without increasing the experimental error.

Conclusions

Results of this study show the technical and experimental conditions needed to build robust and transferable NIRM spectral libraries on animal feeds to unify all pre-treatment and instrumental parameters, in order to guarantee that spectral information transferability between instruments will be similar.

Statistical results have shown as the optimum experimental parameters a particle size of 1 mm for sample preparation prior to NIRM analysis and an average of at least of 70 scans by particle to minimise the noise in spectral data.

Further research is needed to transfer spectral libraries between NIRM instruments allocated in different laboratories; when using FT-NIR, all sources of variation between instruments are minimised, which make it possible to assay different strategies of spectral transference directly and apply classical standardisation methods.

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