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Prediction of lignin content in ruminant diets and faecal samples using rapid analytical techniques.

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ABSTRACT

The measurement of lignin content in ruminant diet and faecal samples is important for digestibility studies, but it is typically time consuming and costly. The work reported involved correlation of traditional wet chemistry data with that from three rapid instrumental techniques, Fourier Transform Infrared spectroscopy (FTIR), Conventional Thermogravimteric Analysis (TGA) and High Resolution TGA (MaxRes TGA) to predict lignin content of diets and faeces from digestibility trials. Calibration and performance data indicated that the FTIR model was acceptable for screening whilst the Conventional and MaxRes TGA predictions were of high accuracy for quantitative analysis. Cross validation and model performance data revealed that MaxRes TGA provided the best performing predictive model. This work showed that MaxRes TGA can accurately predict lignin content in ruminant diet and faecal samples with distinct advantages over traditional wet chemistry, namely the requirement for small sample size, ease of sample preparation, speed of analysis and high sample throughput at considerably lower cost.

KEYWORDS

Lignin prediction, TGA, MaxRes TGA, FTIR, diets, faeces

INTRODUCTION

Plant cell walls contain structural bio-macromolecules in the form of cellulose, hemicellulose, lignin and pectins, in variable ratios dependent on tissue type, growth stage, harvest period, plant species and external factors such as environmental conditions, biotic and abiotic stress. [1,2] The presence of lignin in the plant cell wall is associated with structural integrity, protection against damage and stress tolerance. [3,4] Lignins have a complex molecular structure with a variety of inter-monomer linkages [5] and cross-linking between the polysaccharides (cellulose and hemicellulose) and lignin via ester and ether linkages [6] both of which inhibit forage digestibility in ruminants. [7] The quantification of lignin is difficult not only because of its varying monomeric composition but also because lignins are covalently linked with cell wall carbohydrates, proteins, phenolics, or other compounds [8] that may also affect digestibility.

Determination of lignin in animal feeds and forages is important as higher lignin content is generally associated with lower digestibility, leading to lower voluntary intakes [9,10] and reduced animal performance levels. Historically, lignin has been determined by gravimetric [11] or spectrophotometric [12] methods, both of which are time-consuming and costly, requiring lengthy wet chemical sample preparation techniques. Many other analytical methods have been utilised to determine the lignin content of plant lignocellulosic biomasses, in many cases aided by the availability of powerful multivariate analysis tools which allow accurate prediction models to be created. Techniques such as tissue colour difference following selective staining, [13] the thioglycolic acid method, [14] Near Infrared Spectroscopy, [15] Fourier-transform Mid-Infrared Spectroscopy, [16] solid state Nuclear Magnetic Resonance, [17] Thermogravimetric Analysis, [18] Analytical Pyrolysis [19] and Fourier-transform Raman Spectroscopy [20] have been reported in the literature as alternatives to the traditional gravimetric and spectrophotometric methods.

The availability of not only rapid high throughput but also technologies with a high degree of accuracy, which are widely applicable to measure lignin content in plant materials, is advantageous both for the scientific community and industry, where large sample numbers need to be analysed. For example, forage grass breeders, those processing lignocellulose (paper and pulping), the biomass for energy industry and animal feed producers, would benefit from more cost effective and less time consuming methods to estimate lignin content in feedstock materials. Our approach involved initial measurement of lignin in diet and faecal samples from sheep digestibility trials using a traditional wet chemistry technique. These diet and faecal samples were also analysed using three rapid instrumental techniques, namely Fourier Transform Infrared Spectroscopy (FTIR), Conventional Thermogravimetric Analysis (TGA) and High Resolution TGA (MaxRes TGA). FTIR spectroscopy is an established technique that provides detailed sample chemical information and can be used to characterise a range of components within samples. [21,22] It can also be used for quantitative prediction of sample components. [9,23] Conventional TGA uses constant heating profiles to determine decomposition rates of materials and compositional analysis. The decomposition steps associated with thermal degradation of plant biomass are not clearly separated and overlap with poor resolution. However this can be improved by lowering the heating rate during the decomposition steps using Hi-Res [24] or MaxRes TGA methods resulting in a significant signal change. This increases the resolution of decomposition measurement because weight loss events that are in close proximity no longer overlap at a low heating rate. The resulting temperature programme is consequently composed of discrete dynamic segments. [25] High resolution TGA has been investigated by a number of authors to study a range of materials including inorganics, [24] polymers and composites, [26] lignocellulosic biomass [27,28] and lignins [29] amongst others. All of these authors reported an improvement in results when Hi-Res TGA was compared with Conventional TGA. The work reported here correlated wet chemical

lignin results with data from each of the instrumental techniques, using Partial Least Squares Regression (PLSR) regression analysis, to create predictive models for lignin determination of ruminant diet and faecal sample sets.

MATERIALS AND METHODS

Diet and Faecal Samples. All *in vivo* procedures were licensed and monitored by the UK government Home Office under the Animal (Scientific Procedures) Act 1986. Diet and faecal samples for calibration development were obtained from two sheep feeding trials, the first at the University of Reading (UoR), UK, and the second at the Agri-Food and Biosciences Institute for Northern Ireland (AFBI), UK. The UoR experiment involved a total of thirty-six wether Texel x Mule sheep in an in vivo digestibility study that has been described previously. [30] In brief, ninety perennial ryegrass (PRG) and clover mixture silages with a range of clover concentrations from 40 to 1000 g/kg DM were each fed to three sheep in multiple 3x3 Latin Square designs with five day diet and faecal collection periods for determination of digestibility. Silages were obtained as large bales or chopped clamp material. Silages were chopped and mixed for uniformity and then frozen in vacuum sealed bags in quantities sufficient for daily feeding. Mature wether sheep were then fed each silage, thawed immediately before feeding, for three week periods (three wethers per silage) with five day total collection of faeces while sheep were housed in digestion crates for the last seven days of each period (two days adaptation to crates followed by five days of faecal collection). Sheep were fed silages for *ad libitum* intakes along with 20 g/d of a mineral and vitamin mixture. Representative daily sub-samples of silages and corresponding total faecal collections were immediately added to a composite frozen sample for each five day collection period. A sample subset of ten of the silages fed and their respective faecal samples (one wether per silage) were selected for instrumental analyses based on low, medium and high clover diet contents to cover a range of silage clover concentrations. The AFBI experiment

involved forty eight pregnant ewes from four breed types (Texel X, Highlander X, Belclare X and Lleyn X) offered three different diets (two grass silage diets and one all concentrate diet). The animals were allocated to three diet treatment groups (n = 16 ewes in each treatment), balanced for breed, live weight and condition score. Two sampling periods (seven days each) were undertaken, each with eight ewes from each diet group (i.e. twenty four ewes in each period). At each sampling period (six consecutive days), ewes were offered either grass silage 1, grass silage 2 or a diet based on concentrate (soya) and 50 g/d of straw (to meet forage requirements). Experimental diets were fed for at least ten days before the start of each sampling period. Silages were fed on an *ad libitum* basis, with the levels of forage designed to ensure a refusal margin of 10%. The total daily concentrate diet allowance was offered in two equal-sized meals at 09.30 and 16.30 daily to minimize the risk of acidosis. A sample of each diet and corresponding faecal sample from each breed fed that diet (three diet and twelve faecal samples) were selected for analysis.

Chemical Analyses. Frozen samples of diet and faeces were thawed and mixed before analysis for nitrogen (N) content by the Kjeldahl method (Tecator Auto 1030 Kjeldahl Analyser, Foss Tecator, Sweden). Oven dried samples (85 °C for 72 h) were used to determine DM content using the volatile corrected oven dry matter (VCODM) method, [31] before milling through a 1 mm screen for the following laboratory analyses. Ash content was measured by combustion of a 3 g sample in a muffle furnace at 600 °C for 6 h. Neutral Detergent Fiber (NDF) and Acid Detergent Fiber (ADF) were determined sequentially without amylase [32] and all values were expressed exclusive of residual ash. Acid detergent lignin (ADL) content was determined by solubilisation of cellulose with sulphuric acid. [11]

Conventional TGA. Conventional TGA combustion analysis was performed on a TGA/DSC1 Thermogravimetric Analyser (Mettler Toledo, Switzerland). Dried, milled diet and faeces samples (3 mg) were analysed in triplicate by heating dynamically from room

119 temperature to 600 °C in alumina crucibles (70 µL) at a heating rate of 20 °C min^{-1} under
120 compressed air (BOC, UK) at a flow rate of 30 mL min^{-1}. Peak weight loss (WL), peak
121 combustion temperature (PT) and combustion char residue (RES) characteristics from
122 thermogravimetric and first derivative (*dw/dt*) weight loss curves were evaluated
123 quantitatively using STARe (ver 9.30) Evaluation Software (Mettler Toledo, Switzerland).

124 **MaxRes TGA.** MaxRes TGA was performed using the same sample protocol and
125 equipment as Conventional TGA but under a pyrolytic environment rather than an oxidative
126 one. Pyrolysis was chosen to try to maximise the resolution between weight loss events,
127 which is more difficult to achieve if oxygen is present in the reactive gas. Samples were
128 heated from room temperature to 600 °C using a sample weight loss modulated variable
129 heating rate programme set up in the MaxRes Analysis Toolkit. A dynamic heating rate of 10
130 °C min^{-1} was imposed from 40 - 125 °C. From 125 - 600 °C the base heating rate of 10 °C
131 min^{-1} was adjusted as follows: as the rate of weight loss exceeded 3 µg s^{-1} the heating rate
132 was halved, if after a 12 s timeout the rate of weight loss still exceeded 3 µg s^{-1} the heating
133 rate was halved again and so on to a minimum of 0.5 °C min^{-1}. As the rate of weight loss fell
134 below 1 µg s^{-1} the heating rate doubled, if after a 12 s timeout the rate of weight loss
135 remained below 1 µg s^{-1} the heating rate doubled again and so on until a maximum rate of 20
136 °C min^{-1} was reached. Samples were pyrolysed under nitrogen at a flow rate of 30 mL min^{-1}
137 and similar data to the Conventional TGA runs were recorded.

138 **FTIR Spectroscopy.** The FTIR spectra of dried milled diet and faeces samples were
139 obtained using a Spectrum One FTIR Spectrometer (Perkin Elmer Inc., USA) equipped with
140 an attenuated total reflectance sampling device containing a diamond/ZnSe crystal. Spectra
141 were scanned at room temperature in transmission mode (%T) between 4000-650 cm^{-1}, with
142 50 scans at a scan speed of 0.20 cm s^{-1} and a resolution of 4 cm^{-1}. Samples were scanned in
143 triplicate to obtain an average spectrum and the background spectrum was scanned under the

same instrumental conditions. The spectra were acquired using Spectrum Software (version 10.4.1, Perkin Elmer Inc., USA), then base line corrected and normalised.

Statistical Analysis and Data Modelling. Diet and faeces chemical composition results, Conventional TGA and MaxRes weight loss data were analysed using Microsoft Excel to calculate Minima (Min), Maxima (Max) Mean and Standard Deviation (Sdev). Diet and faecal results for lignin contents measured by wet chemistry, continuous weight loss data from the Conventional and MaxRes TGA analyses, along with spectral absorbance data from FTIR analysis, were exported to "The Unscrambler" multivariate statistical software package (CAMO, Norway) for Principal Component Analysis (PCA) and Partial Least Squares Regression (PLSR) calibration development. PCA is a projection method that helps to visualize all of the information contained in a data table. PCA estimates in what respect one sample is different from another, which variables contribute most to this difference, and whether those variables contribute in the same way (i.e. are correlated) or independently from each other. It also enables detection of sample patterns, like any particular grouping. PLSR models both the X- and Y-variables in a data set simultaneously to find the latent (or hidden) variables in X that will best predict the latent variables in Y. PLSR maximizes the covariance between X and Y. PLSR was performed using the guidelines presented in "The Unscrambler" user manual and the calibration models generated were full sized, data were centred and the suggested number of principle components for each was nine.

Because the sample set was relatively small, full cross validation was employed to facilitate maximum use of the available data for validation of the calibrations. Using full cross validation present in the Unscrambler, one sample was taken out of the calibration data set and the model was calibrated on the remaining data points. Then the values for the left-out sample were predicted and prediction residuals computed. The process was repeated by leaving out a different sample until every sample had been left out once. All prediction

residuals were then combined to compute the validation residual variance. Calibration precision was evaluated by the coefficient of determination for calibration (R^2c) and standard error of calibration (SEC). Predictive ability of calibrations [33] was internally evaluated by coefficient of determination for cross validation (R^2cv) and standard error of cross-validation (SECV). Calibration performance of the models was assessed using the RPD value defined as the ratio of performance to deviation. RPD value was based on the interpretation presented in [34] where an RPD value of < 2.0 is not suitable for prediction; values between 2.0 – 2.4 are acceptable for screening purposes; values between 2.5 – 2.9 are useful for quantification; and values ≥ 3.0 indicate high accuracy for quantitative analysis.

RESULTS AND DISCUSSION

Composition of diet and faecal samples. Chemical composition of diet and faecal samples is represented in **Table 1**. UOR diets were more variable than those of the AFBI set with wider ranges for all parameters measured with the exception of DM. The concentrate diet was responsible for highest DM in the AFBI samples. The higher ADL and lower N content of the UoR diets could be attributed to the inclusion of a range of clover concentrations in these diets mixed with PRG. Faecal samples from both sets exhibited higher ash and ADL content when compared to diet samples. This would be expected due to the removal of digestable materials i.e. cellulose and hemicellulose, leaving indigestible components such as lignin and inorganic fractions as relatively larger proportions of the faeces. The means and ranges of DM for faeces were similar to their diets. The results indicated that the composition of diet and faecal sample sets were diverse and importantly, an extensive range of lignin contents were present in the samples for further analysis. It is also possible to infer that the AFBI diets were of higher potential digestibility when levels of DM, Ash and ADL were compared in the faecal samples from both sets of studies.

193 **Conventional TGA.** The first derivative thermogravimetric (DTG) weight loss curves
194 of diet and faecal samples are presented in **Figure 1**. For the diet samples, two main
195 combustion events occurred around 300 °C and 450 °C. Two main combustion events were
196 also noted for faecal samples at temperatures close to 320 °C and 490 °C with these events
197 corresponding to biopolymer decomposition and char combustion respectively. The pyrolysis
198 of cellulose has two temperature dependant pathways which can result in formation of
199 carbonyl, carboxyl and hydroperoxide groups and the evolution of a range of low molecular
200 weight products including CO, CO_2 and anhydrosugars whereas lignin is mainly charred to a
201 carbonaceous residue [35]. The samples presented in **Figure 1** displayed a range of lignin
202 contents (diets 1.4 – 9.0%, faeces 7.2 – 19.9%) representing the variation in weight loss
203 profiles in samples from the study and specific plots for individual samples have been
204 depicted.

205 Weight loss (WL), peak combustion temperature (PT) and combustion char residue
206 (RES) data for diet and faecal samples are displayed in **Table 2**. Mean WL 1 for UoR diets
207 was higher than the AFBI samples, whilst PT1, WL 2 and PT 2 data were similar for both
208 diet sets. Mean RES value was higher for the UoR diets. AFBI faecal samples had higher
209 mean WL 1 and WL 2 values but similar PT 1 and PT 2 to those from UoR, and the mean
210 RES value for UoR was twice that of the AFBI samples. The peak shoulders and split peaks
211 (100 – 350 °C and 400 – 450 °C) which were present on the diet DTG weight loss profiles
212 (**Figure 1a**), were either not as pronounced or are not present at all on the faecal samples
213 (**Figure 1b**). This is because much of the hemicellulose and cellulose present in the diets had
214 been degraded during ruminant digestion [36] and this is also reflected by temperature shifts
215 from 300 to 320 °C for PT 1 and 450 to 490 °C for PT 2 from diet to faecal analyses for both
216 data sets. The higher PT's for the faecal samples were due to the relatively increased lignin
217 content in comparison to the diets, which shifted the thermal degradation to higher

218 temperatures. Cellulose content may also enhance the combustion characteristics and
219 decomposition of lignin [37]. Using model compounds [38] showed cellulose decomposes as a
220 single peak due to its linear structure whereas hemicellulose and lignin decompose over wide
221 temperature ranges due to their branched structures with peak temperatures in the order
222 cellulose < hemicellulose < lignin. Variations in the shape, weight loss characteristics and
223 peak combustion temperatures of diet and faecal samples are directly related to their chemical
224 composition and in particular to their fiber fraction content. [39,40]

225 **MaxRes TGA.** MaxRes DTG pyrolysis weight loss curves of diets and corresponding
226 faeces for individual representative samples with a range of lignin contents are illustrated in
227 **Figure 2**. Diet and faecal samples produced four distinctive decomposition peaks between
228 130 – 350 °C that were associated with fiber fraction content. The decomposition peak at 130
229 °C was a common artefact due to a ramp up in the temperature of the furnace. The
230 decomposition peaks at higher temperatures displayed variable height and width
231 characteristics. These characteristics were expressed as WL, PT and RES data for diet and
232 faecal samples and the quantitative data for the three largest decomposition peaks are
233 presented in **Table 3**. Mean WL, PT and RES values for UoR and AFBI diets were generally
234 similar, with the exception of WL 3 being higher for AFBI diets. The WL 4 on **Table 3** was
235 attributed to sample weight loss between the last major decomposition peak and the end
236 temperature of 600 °C. Comparable trends were noted for the UoR and AFBI faecal samples
237 for mean WL and PT data, and again WL 3 was higher for AFBI samples, while RES was
238 higher for the UoR samples (**Table 3**). Shifts to higher PT's were observed for the major
239 decomposition peaks from the MaxRes analysis as had been found for the Conventional TGA
240 combustions runs. The faecal peak temperatures were all higher than their corresponding
241 diets with increases of 30 – 40 °C recorded due to the increased lignin content in comparison
242 to the diets.

The improved peak resolution when utilising MaxRes TGA compared to conventional TGA, if linked to mass spectrometry, may provide an opportunity to elucidate any variations in structure between samples, or modifications to the biopolymers during digestion, by measurement of the volatile gases evolved. This could be particularly applicable to lignin due to the specificity of evolved gases such as the monomers which comprise the lignin structure. In this study the lignin chemical structures were assumed to be relatively uniform across samples since the diets are composed of the same initial material i.e. perennial ryegrass/clover silages or mixtures of both. Therefore it is expected that lignin structure had little impact in variations in analyses which are instead solely dependent on lignin content. However, if necessary for example where a more varied range of diets were analysed, the incorporation of C^{13} NMR analysis would allow further elucidation of lignin structures [45] from which it may be possible to correlate to MaxRes degradation peaks and indeed evolved gases if the MaxRes TGA is linked to mass spectrometry providing a complementary analysis.

FTIR Spectroscopy. Comparisons of the FTIR spectra of diets and faeces for individual representative samples with a range of lignin contents are presented in **Figure 3**. Major spectral bands were present at 3335 and 1040 cm^{-1} with antisymetric and symmetric vibration peaks observed from 2930 - 2850 cm^{-1}. The prominent peak at 1756 cm^{-1} in the diets had shifted to 1732 cm^{-1} in the faecal samples. A range of peaks between 1640 - 1620 cm^{-1}, 1550 - 1420 cm^{-1}, 1375 - 1310 cm^{-1} and 1240 - 1025 cm^{-1} were common in diet and faecal samples. Peak shifts and differences in peak intensities were noted when comparing the diets and faeces, and the peak at 873 cm^{-1} was more prominent and of greater intensity in the faecal samples. There was a notable reduction in the peak intensity at 1756 cm^{-1} in diet samples compared to the corresponding peak at 1732 cm^{-1} in the faeces, with differences in

267 the number of peaks and their intensities also noted for the spectral bands from 1640 - 1025
268 cm^{-1}.

269 Major diet spectral bands at 3335 cm^{-1} and 1040 cm^{-1} (OH and C-O stretches) and
270 peaks from 2930 - 2850 cm^{-1} were associated with cellulose. [22] A peak at 2850 cm^{-1} (C-H
271 stretch) and the peak at 1732 cm^{-1} were due to the presence of lignin and hemicellulose
272 respectively. [21,22] Hemicellulose peaks were present at 1640 - 1620 cm^{-1}, bands between 1550
273 - 1420 cm^{-1} (aromatic C=C, C-H bends and C-H deformations) and at 1240 cm-1 (aromatic
274 C-O-C stretch) were from lignin, peaks between 1375 - 1310 cm^{-1} (C-H asymmetric
275 deformation) and a peak/shoulder at 873 cm^{-1} were associated with cellulose. [21] Reduced
276 intensity at 1732 cm^{-1} indicated lower faecal hemicellulose. [22] The variation in spectral
277 intensities and peak shifts from 1640 - 1172 cm^{-1} were due to degradation of dry matter,
278 particularly hemicellulose and cellulose during digestion, and the relative increase in faecal
279 lignin concentration due to its poor digestibility. [41]

280 **Statistical Analysis and Data Modelling.** Calibration and validation statistics along
281 with RPD values for the predictive models for each analytical technique are displayed in
282 **Table 4**. The calibration and cross validation data for models derived from each technique
283 indicated excellent precision and predictive ability, and that all models could be used to
284 predict lignin content of the diet and faecal samples. Best calibration statistics were obtained
285 using the Conventional TGA data (R^2c 0.98 and SEC 1.08) while best cross validation
286 statistics were produced from the MaxRes TGA results (R^2cv 0.95 and SECV 1.64).
287 Calibration performance of the prediction equations was assessed using the RPD value,
288 which revealed that the FTIR model (RPD 2.20) did not perform as well as the Conventional
289 and MaxRes TGA models (RPD 3.05 and 3.38 respectively). Using the interpretation
290 presented in, [34] the FTIR prediction model would be acceptable for sample screening while
291 the Conventional and MaxRes TGA predictions could be regarded as highly accurate and

could be used for quantitative analysis. The combination of best cross validation statistics and highest RPD value indicated that the MaxRes TGA technique provided the best performing predictive model of the three rapid analytical methods studied. Multivariate analysis results represented by PCA and calibration model regression coefficients are displayed in **Figure 4a** and **Figure 4b** respectively. PCA of MaxRes TGA continuous weight loss data (**Figure 4a**) produced two distinct clusters for diet and faecal samples based on the differences in chemical composition, including lignin content. The first two principle components explained 92% of the sample variation. While it is accepted that lignin degradation occurs across a wide temperature range, the calibration model regression coefficients (**Figure 4b**) showed the most influential data points along the temperature scale for lignin prediction (160 – 490 °C). Regression coefficients show how each variable is weighted when predicting a particular Y response. In a regression model equation, regression coefficients are the numerical coefficients that express the link between variation in the predictors and variation in the response. The data points corresponded with the areas of maximum weight loss on MaxRes DTG thermograms (**Figure 2**) and were consistent with the view that thermal degradation of lignin involves multi-step reactions that occur over a temperature range. [29]

Other studies have used Near Infrared Spectroscopy (NIRS) to quantify lignin content in similar samples. The calibration produced by [42] was of poorer quality (R^2cv 0.77; RPD 2.1) than those developed in our work, while those reported by [43,44] could be regarded as being of similar or better quality (R^2cv 0.94; RPD 4.0 and R^2cv 0.95; RPD 4.4 respectively) than ours. All of these studies used larger sample sets (n = 84-299). A Hi-Res TGA calibration model created for lignin prediction in willow biomass [28] reported an R^2c of 0.76, while lignin calibrations developed by [1] for switchgrass using FTIR and Pyrolysis Molecular Beam Mass Spectrometry (PyMBMS), reported statistics of R^2cv 0.96 and R^2cv 0.94 respectively. In conclusion, this work has demonstrated that the three rapid analytical technologies

investigated can all accurately predict lignin content of ruminant diet and faecal samples. They have distinct advantages over many of the conventional methodologies in use, namely the requirement for small sample size, ease of sample preparation, automation, speed of analysis and high sample throughput at considerably lower cost. The reported research has given an initial indication of the positive potential for the techniques studied to predict lignin content of a relatively small sample set. Increasing the diet and faecal sample set sizes should improve the statistics and predictive ability of the equations that were developed. Follow on work to look at a wider range of diets and corresponding faecal samples and to externally validate the models with blind samples would be important for future application of the approaches. The addition of a mass spectrometer to the TGA equipment using the MaxRes method for evolved gas analysis, would enable detailed characterisation of resolved peaks, increasing the understanding of the thermal decomposition of biomass under pyrolytic conditions.

ABBREVIATIONS USED

ADF – Acid Detergent Fiber

AFBI – Agri-Food and Biosciences Institute

DM – Dry Matter

DTG – Derivative Thermogravimetry

FTIR – Fourier Transform Infrared

Hi-Res TGA – High Resolution Thermogravimetric Analysis

Max-Res TGA – Maximum Resolution Thermogravimetric Analysis

Max – Maximum value

339 Min – Minimum value

340 NDF – Neutral Detergent Fiber

341 PCA – Principle Component Analysis

342 PLSR – Partial Least Squares Regression

343 PRG - Perennial Ryegrass

344 PT – Peak Temperature

345 RES – TGA Combustion Char Residue

346 RPD – Ratio of Prediction to Deviation

347 R^2c - Coefficient of determination for calibration

348 R^2cv - Coefficient of determination for cross validation

349 Sdev – Standard Deviation

350 SEC – Standard Error of Calibration

351 SECV – Standard Error of Cross Validation

352 TGA – Thermogravimetric Analysis

353 UoR – University of Reading

354 WL – Thermogravimetric Weight Loss

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358

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FIGURE CAPTIONS

Figure 1. Conventional TGA derivative thermogravimetric (DTG) plots of weight loss profiles (*dw/dt*) against increasing temperature for representative samples of (a) diets and (b) faeces with a range of lignin contents indicated by coloured lines.

Figure 2. MaxRes TGA derivative thermogravimetric (DTG) plots of weight loss profiles (*dw/dt*) against increasing temperature for representative samples of (a) diets and (b) faeces with a range of lignin contents indicated by coloured lines.

Figure 3. FTIR spectra for representative samples of (a) diets and (b) faeces with a range of lignin contents indicated by coloured lines. Plots show percentage transmittance (%T) against change in wavelength (cm^{-1}) with major peak markers included.

Figure 4. Multivariate analysis of MaxRes TGA continuous weight loss data showing (a) Principle Component Analysis with diet and faecal samples separating in to two distinct clusters and (b) calibration model regression coefficients showing the most important data points along the temperature scale for lignin prediction with both positive and negative influences.

Table 1. Chemical Composition of Diet and Faecal Samples Used to Develop Calibration Equations From Sheep Feeding Trials at UoR and AFBI. ADL Content of Diet and Faecal Samples Used for Calibration Validation is Also Shown. Minima, Maxima, Means and Standard Deviations are Presented.

Parameter	Min	Max	Mean	Sdev	Min	Max	Mean	Sdev
		UoR Diets (n=10)				AFBI Diets (n=3)		
DM (g/kg)	222.0	592.1	410.0	148.6	299.4	872.4	511.4	314.2
Ash (g/kg DM)	68.6	128.3	98.4	27.2	89.3	103.0	94.6	7.4
N (g/kg DM)	4.2	17.5	9.2	4.5	20.8	27.6	24.3	3.4
ADF (g/kg DM)	228.6	386.3	323.0	47.5	322.9	372.1	343.1	25.7
NDF (g/kg DM)	299.2	521.5	419.1	80.1	552.2	577.5	563.9	12.8
ADL (% DM)	2.1	9.0	5.4	2.4	1.4	2.5	2.1	0.5
		UoR Faeces (n=10)				AFBI Faeces (n=12)		
DM (g/kg)	222.0	671.2	422.3	144.6	227.6	363.7	293.7	41.3
Ash (g/kg DM)	90.3	282.9	181.6	70.6	93.9	209.6	133.4	39.7
ADL (% DM)	11.1	19.9	15.4	3.5	7.2	15.5	12.0	3.1

Table 2 Combustion TGA Results Showing Weight Loss (WL) and Peak Temperature (PT) for Each of Two Steps Along With Residue (RES) Data for Diet and Faecal Samples From the UoR and AFBI Trials. Minima, Maxima, Means and Standard Deviations are Presented.

Parameter	Min	Max	Mean	Sdev	Min	Max	Mean	Sdev
		UoR Diets (n=10)				AFBI Diets (n=3)		
WL1 (%)	39.8	53.8	46.8	4.3	37.6	44.0	41.5	3.4
PT 1 (°C)	278	325	302	11.5	293	323	304	16.1
WL 2 (%)	25.8	31.6	29.3	2.2	27.0	33.2	29.5	3.2
PT 2 (°C)	439	475	452	13.1	451	460	454	5.2
RES (%)	9.8	17.5	14.1	2.5	5.0	13.9	10.3	4.7
		UoR Faeces (n=10)				AFBI Faeces (n=12)		
WL 1 (%)	40.4	55.7	48.5	5.7	48.7	59.0	53.9	3.2
PT 1 (°C)	308	336	323	8.7	309	337	325	4.4
WL 2 (%)	9.2	22.7	15.3	4.2	8.4	22.6	16.5	4.9
PT 2 (°C)	442	510	488	22.7	481	506	492	6.1
RES (%)	12.8	29.8	20.7	5.8	7.1	25.5	10.5	5.0

Table 3. MaxRes Pyrolysis TGA Results Showing Weight Loss (WL) and Peak Temperature (PT) Individual Steps Indicated by Integer Along With Char Residue (RES) Data for Diet and Faecal Samples From the UoR and AFBI Trials. Minima, Maxima, Means and Standard Deviations are Presented.

Parameter	Min	Max	Mean	Sdev	Min	Max	Mean	Sdev
		UoR Diets (n=10)				AFBI Diets (n=3)		
WL1 (%)	9.5	17.1	12.4	2.0	10.8	12.6	11.8	0.9
PT 1 (°C)	212	257	248	13.1	207	260	242	30.0
WL2 (%)	10.4	24.3	18.0	4.5	13.7	21.9	16.8	4.4
PT 2 (°C)	262	293	285	8.8	261	291	281	16.8
WL3 (%)	7.2	18.4	9.9	3.3	6.2	19.9	14.0	7.0
PT 3 (°C)	293	322	314	8.2	291	338	312	19.4
WL4 (%)	14.3	17.9	15.9	1.4	13.6	18.4	15.8	2.4
RES (%)	37.2	42.5	39.9	1.4	37.0	41.5	38.5	2.6
		UoR Faeces (n=10)				AFBI Faeces (n=12)		
WL1 (%)	12.8	19.1	15.0	2.2	11.9	17.2	14.9	2.0
PT 1 (°C)	282	294	288	3.8	278	287	282	2.4
WL2 (%)	11.6	22.2	17.1	3.8	11.0	20.6	16.8	3.3
PT 2 (°C)	310	326	318	5.5	306	318	312	3.2
WL3 (%)	6.6	9.2	7.7	1.0	7.3	14.1	10.1	2.6
PT 3 (°C)	349	358	354	3.7	340	347	342	2.2
WL4 (%)	12.6	20.0	16.2	2.9	13.6	16.4	15.0	0.9
RES (%)	39.6	53.7	45.7	4.9	37.1	48.1	41.0	3.5

Table 4. Calibration (R^2c, SEC) and Cross Validation (R^2cv, SECV) Statistics for Prediction Models Developed From the Three Analytical Methods (FTIR, Conventional and MaxRes TGA). Calibration Performance was Measured by Calculation of the RPD Value.

Method	n	Calibration		Cross Validation		
		R^2c	SEC	R^2cv	SECV	RPD
FTIR	35	0.97	1.42	0.89	2.52	2.20
Conventional TGA	35	0.98	1.08	0.94	1.84	3.05
MaxRes TGA	34	0.98	1.17	0.95	1.64	3.38

Figure 1. **To be published in colour**

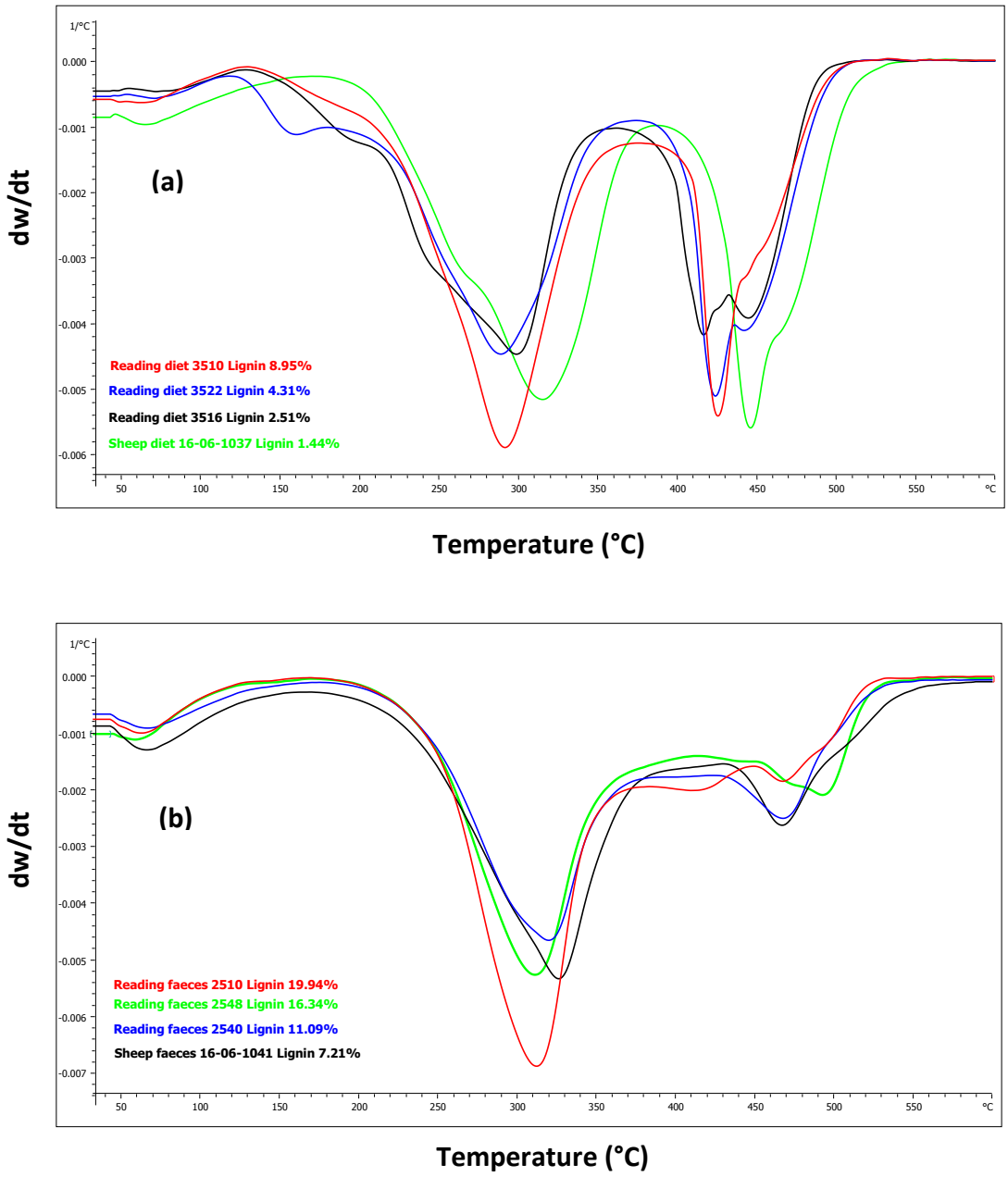


Figure 2. **To be published in colour**

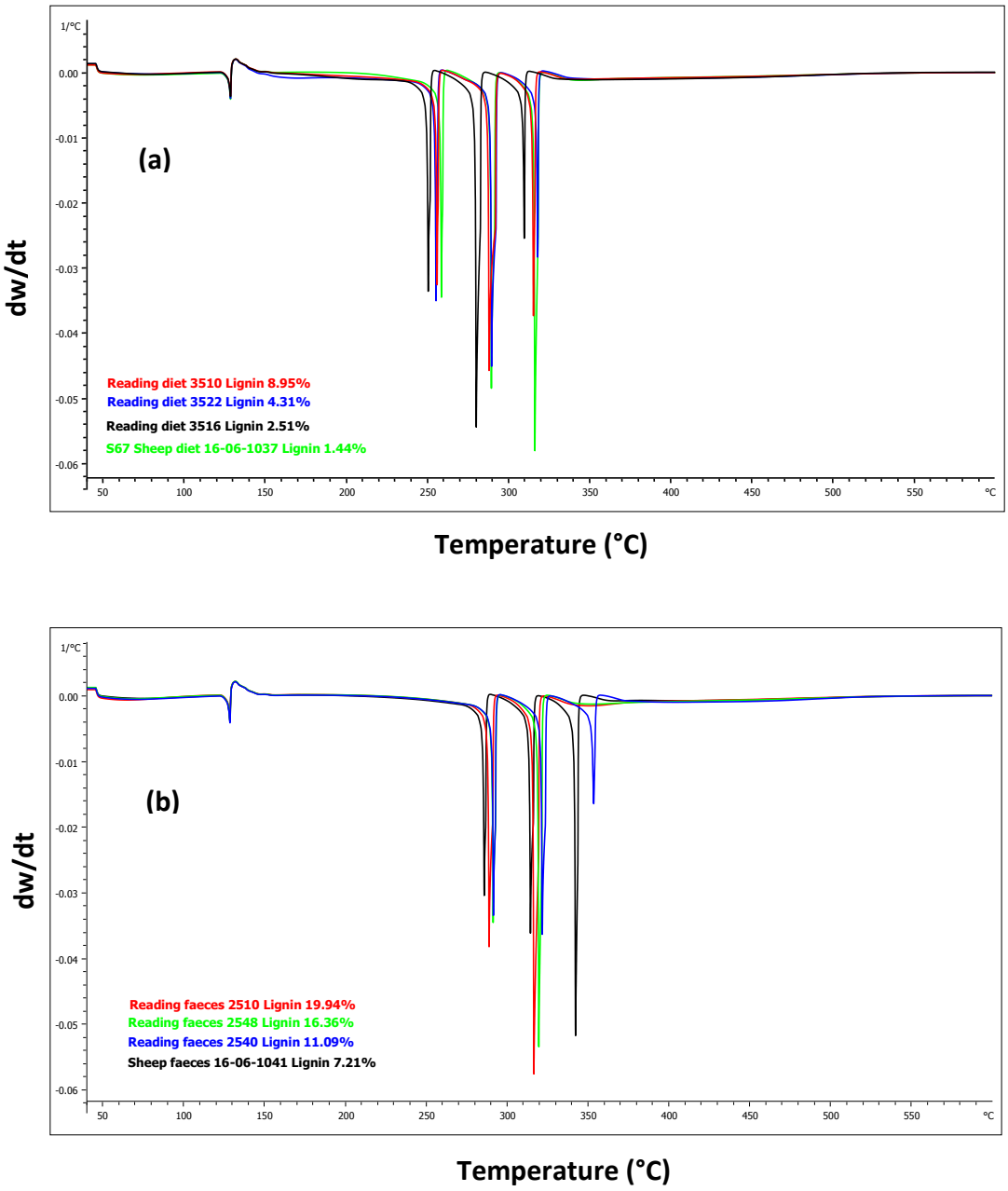


Figure 3. **To be published in colour**

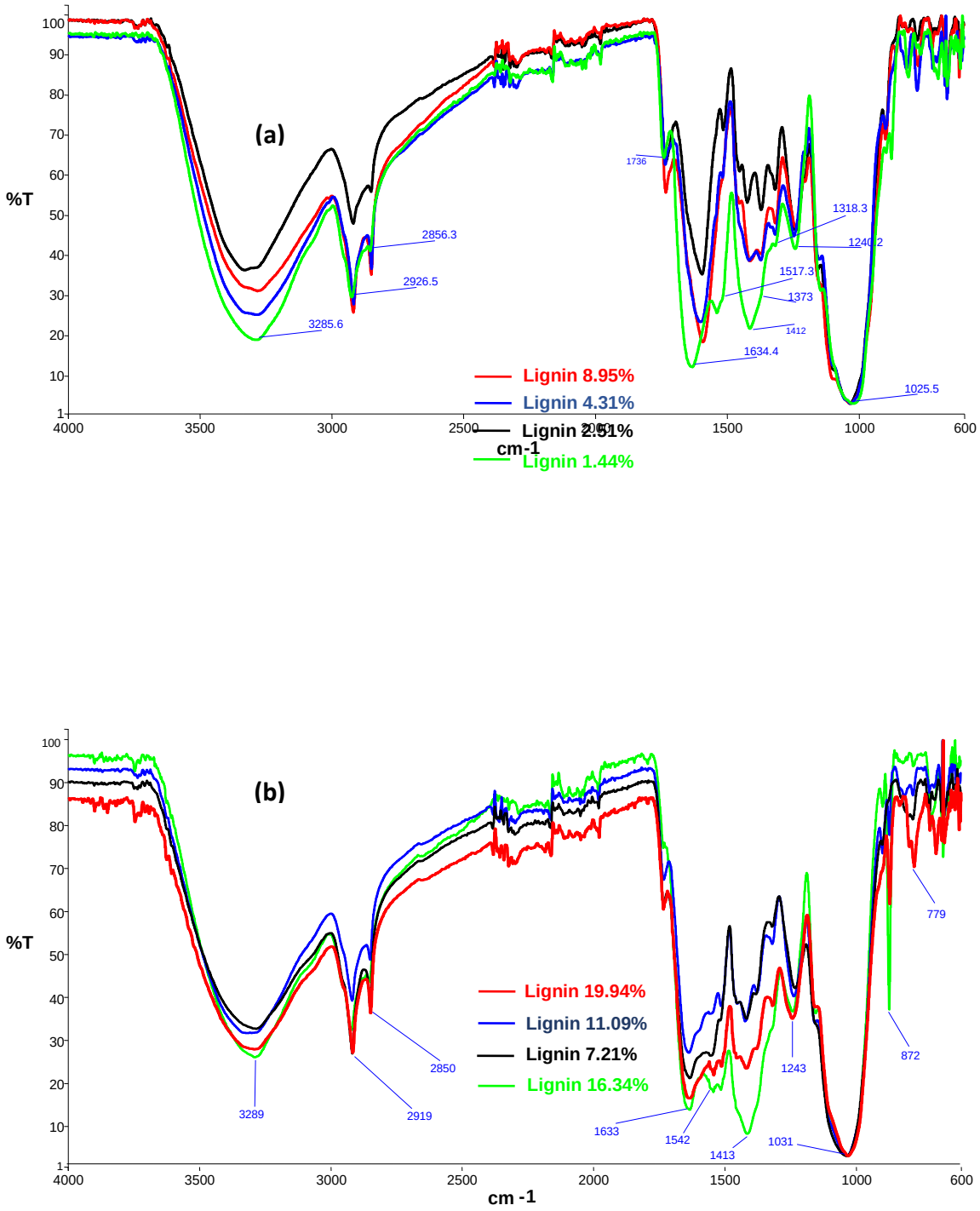
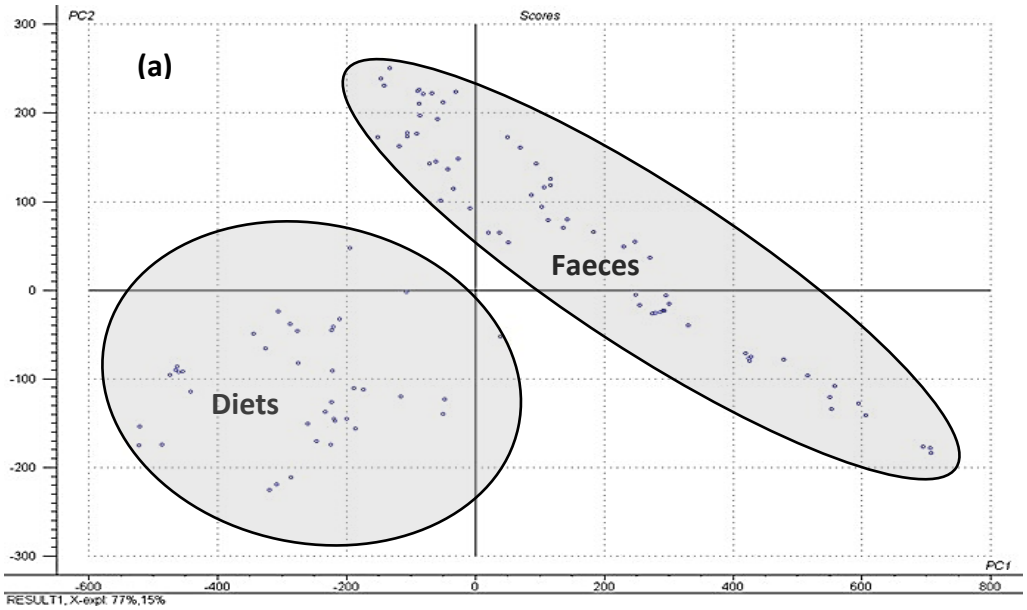
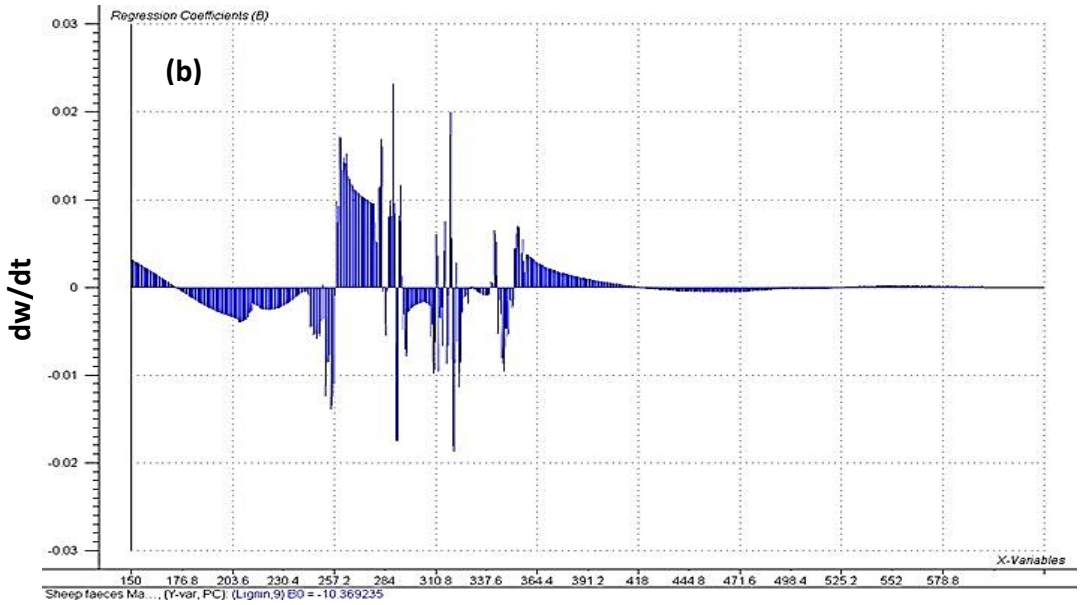


Figure 4.

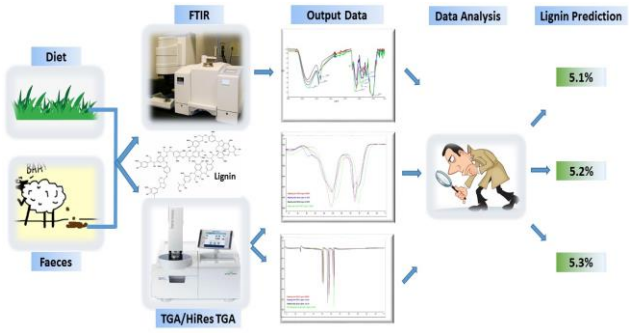




Temperature (°C)

TOC Graphic

Prediction of lignin content in ruminant diets and faecal samples using rapid analytical techniques.



TOC categories:

1. Analytical Methods
2. Agricultural and Environmental Chemistry
3. Biofuels and Biobased Products