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Accepted Version

Lyons, G., Carmichael, E., McRoberts, C., Aubrey, A., Thomson, A. and Reynolds, C. K. ORCID: <https://orcid.org/0000-0002-4152-1190> (2018) Prediction of lignin content in ruminant diets and faecal samples using rapid analytical techniques. *Journal of Agricultural and Food Chemistry*, 66 (49). pp. 13031-13040. ISSN 0021-8561 doi: <https://doi.org/10.1021/acs.jafc.8b03808> Available at <https://centaur.reading.ac.uk/80754/>

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To link to this article DOI: <http://dx.doi.org/10.1021/acs.jafc.8b03808>

Publisher: American Chemical Society

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

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1 **ABSTRACT**

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

15

16 **KEYWORDS**

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

18

19 INTRODUCTION

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

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

44 The availability of not only rapid high throughput but also technologies with a high
45 degree of accuracy, which are widely applicable to measure lignin content in plant materials,
46 is advantageous both for the scientific community and industry, where large sample numbers
47 need to be analysed. For example, forage grass breeders, those processing lignocellulose
48 (paper and pulping), the biomass for energy industry and animal feed producers, would
49 benefit from more cost effective and less time consuming methods to estimate lignin content
50 in feedstock materials. Our approach involved initial measurement of lignin in diet and faecal
51 samples from sheep digestibility trials using a traditional wet chemistry technique. These diet
52 and faecal samples were also analysed using three rapid instrumental techniques, namely
53 Fourier Transform Infrared Spectroscopy (FTIR), Conventional Thermogravimetric Analysis
54 (TGA) and High Resolution TGA (MaxRes TGA). FTIR spectroscopy is an established
55 technique that provides detailed sample chemical information and can be used to characterise
56 a range of components within samples.^{21,22} It can also be used for quantitative prediction of
57 sample components.^{9,23} Conventional TGA uses constant heating profiles to determine
58 decomposition rates of materials and compositional analysis. The decomposition steps
59 associated with thermal degradation of plant biomass are not clearly separated and overlap
60 with poor resolution. However this can be improved by lowering the heating rate during the
61 decomposition steps using Hi-Res²⁴ or MaxRes TGA methods resulting in a significant
62 signal change. This increases the resolution of decomposition measurement because weight
63 loss events that are in close proximity no longer overlap at a low heating rate. The resulting
64 temperature programme is consequently composed of discrete dynamic segments.²⁵ High
65 resolution TGA has been investigated by a number of authors to study a range of materials
66 including inorganics,²⁴ polymers and composites,²⁶ lignocellulosic biomass^{27,28} and lignins
67²⁹ amongst others. All of these authors reported an improvement in results when Hi-Res TGA
68 was compared with Conventional TGA. The work reported here correlated wet chemical

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

72 MATERIALS AND METHODS

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

94 involved forty eight pregnant ewes from four breed types (Texel X, Highlander X, Belclare X
95 and Lleyn X) offered three different diets (two grass silage diets and one all concentrate diet).
96 The animals were allocated to three diet treatment groups (n = 16 ewes in each treatment),
97 balanced for breed, live weight and condition score. Two sampling periods (seven days each)
98 were undertaken, each with eight ewes from each diet group (i.e. twenty four ewes in each
99 period). At each sampling period (six consecutive days), ewes were offered either grass silage
100 1, grass silage 2 or a diet based on concentrate (soya) and 50 g/d of straw (to meet forage
101 requirements). Experimental diets were fed for at least ten days before the start of each
102 sampling period. Silages were fed on an *ad libitum* basis, with the levels of forage designed
103 to ensure a refusal margin of 10%. The total daily concentrate diet allowance was offered in
104 two equal-sized meals at 09.30 and 16.30 daily to minimize the risk of acidosis. A sample of
105 each diet and corresponding faecal sample from each breed fed that diet (three diet and
106 twelve faecal samples) were selected for analysis.

107 **Chemical Analyses.** Frozen samples of diet and faeces were thawed and mixed
108 before analysis for nitrogen (N) content by the Kjeldahl method (Tecator Auto 1030 Kjeldahl
109 Analyser, Foss Tecator, Sweden). Oven dried samples (85 °C for 72 h) were used to
110 determine DM content using the volatile corrected oven dry matter (VCODM) method,³¹
111 before milling through a 1 mm screen for the following laboratory analyses. Ash content was
112 measured by combustion of a 3 g sample in a muffle furnace at 600 °C for 6 h. Neutral
113 Detergent Fiber (NDF) and Acid Detergent Fiber (ADF) were determined sequentially
114 without amylase³² and all values were expressed exclusive of residual ash. Acid detergent
115 lignin (ADL) content was determined by solubilisation of cellulose with sulphuric acid.¹¹

116 **Conventional TGA.** Conventional TGA combustion analysis was performed on a
117 TGA/DSC1 Thermogravimetric Analyser (Mettler Toledo, Switzerland). Dried, milled diet
118 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

144 same instrumental conditions. The spectra were acquired using Spectrum Software (version
145 10.4.1, Perkin Elmer Inc., USA), then base line corrected and normalised.

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

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

169 residuals were then combined to compute the validation residual variance. Calibration
170 precision was evaluated by the coefficient of determination for calibration (R^2c) and standard
171 error of calibration (SEC). Predictive ability of calibrations³³ was internally evaluated by
172 coefficient of determination for cross validation (R^2cv) and standard error of cross-validation
173 (SECV). Calibration performance of the models was assessed using the RPD value defined as
174 the ratio of performance to deviation. RPD value was based on the interpretation presented in
175 ³⁴ where an RPD value of < 2.0 is not suitable for prediction; values between $2.0 - 2.4$ are
176 acceptable for screening purposes; values between $2.5 - 2.9$ are useful for quantification; and
177 values ≥ 3.0 indicate high accuracy for quantitative analysis.

178 **RESULTS AND DISCUSSION**

179 **Composition of diet and faecal samples.** Chemical composition of diet and faecal
180 samples is represented in **Table 1**. UOR diets were more variable than those of the AFBI set
181 with wider ranges for all parameters measured with the exception of DM. The concentrate
182 diet was responsible for highest DM in the AFBI samples. The higher ADL and lower N
183 content of the UoR diets could be attributed to the inclusion of a range of clover
184 concentrations in these diets mixed with PRG. Faecal samples from both sets exhibited higher
185 ash and ADL content when compared to diet samples. This would be expected due to the
186 removal of digestible materials i.e. cellulose and hemicellulose, leaving indigestible
187 components such as lignin and inorganic fractions as relatively larger proportions of the
188 faeces. The means and ranges of DM for faeces were similar to their diets. The results
189 indicated that the composition of diet and faecal sample sets were diverse and importantly, an
190 extensive range of lignin contents were present in the samples for further analysis. It is also
191 possible to infer that the AFBI diets were of higher potential digestibility when levels of DM,
192 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, CO₂ and anhydrosugars whereas lignin is mainly charred to a
201 carbonaceous residue³⁵. 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 digestion³⁶ 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 lignin³⁷. Using model compounds³⁸ 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.

243 The improved peak resolution when utilising MaxRes TGA compared to conventional
244 TGA, if linked to mass spectrometry, may provide an opportunity to elucidate any variations
245 in structure between samples, or modifications to the biopolymers during digestion, by
246 measurement of the volatile gases evolved. This could be particularly applicable to lignin due
247 to the specificity of evolved gases such as the monomers which comprise the lignin structure.
248 In this study the lignin chemical structures were assumed to be relatively uniform across
249 samples since the diets are composed of the same initial material i.e. perennial
250 ryegrass/clover silages or mixtures of both. Therefore it is expected that lignin structure had
251 little impact in variations in analyses which are instead solely dependent on lignin content.
252 However, if necessary for example where a more varied range of diets were analysed, the
253 incorporation of C¹³ NMR analysis would allow further elucidation of lignin structures ⁴⁵
254 from which it may be possible to correlate to MaxRes degradation peaks and indeed evolved
255 gases if the MaxRes TGA is linked to mass spectrometry providing a complementary
256 analysis.

257 **FTIR Spectroscopy.** Comparisons of the FTIR spectra of diets and faeces for
258 individual representative samples with a range of lignin contents are presented in **Figure 3**.
259 Major spectral bands were present at 3335 and 1040 cm⁻¹ with antisymmetric and symmetric
260 vibration peaks observed from 2930 - 2850 cm⁻¹. The prominent peak at 1756 cm⁻¹ in the
261 diets had shifted to 1732 cm⁻¹ in the faecal samples. A range of peaks between 1640 - 1620
262 cm⁻¹, 1550 - 1420 cm⁻¹, 1375 - 1310 cm⁻¹ and 1240 - 1025 cm⁻¹ were common in diet and
263 faecal samples. Peak shifts and differences in peak intensities were noted when comparing
264 the diets and faeces, and the peak at 873 cm⁻¹ was more prominent and of greater intensity in
265 the faecal samples. There was a notable reduction in the peak intensity at 1756 cm⁻¹ in diet
266 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 $2930 - 2850 \text{ cm}^{-1}$ 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 $1640 - 1620 \text{ cm}^{-1}$, bands between 1550
273 $- 1420 \text{ cm}^{-1}$ (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 $1375 - 1310 \text{ cm}^{-1}$ (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 $1640 - 1172 \text{ cm}^{-1}$ 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^2_c 0.98 and SEC 1.08) while best cross validation
286 statistics were produced from the MaxRes TGA results (R^2_{cv} 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

292 could be used for quantitative analysis. The combination of best cross validation statistics and
293 highest RPD value indicated that the MaxRes TGA technique provided the best performing
294 predictive model of the three rapid analytical methods studied. Multivariate analysis results
295 represented by PCA and calibration model regression coefficients are displayed in **Figure 4a**
296 and **Figure 4b** respectively. PCA of MaxRes TGA continuous weight loss data (**Figure 4a**)
297 produced two distinct clusters for diet and faecal samples based on the differences in
298 chemical composition, including lignin content. The first two principle components explained
299 92% of the sample variation. While it is accepted that lignin degradation occurs across a wide
300 temperature range, the calibration model regression coefficients (**Figure 4b**) showed the most
301 influential data points along the temperature scale for lignin prediction (160 – 490 °C).
302 Regression coefficients show how each variable is weighted when predicting a particular Y
303 response. In a regression model equation, regression coefficients are the numerical
304 coefficients that express the link between variation in the predictors and variation in the
305 response. The data points corresponded with the areas of maximum weight loss on MaxRes
306 DTG thermograms (**Figure 2**) and were consistent with the view that thermal degradation of
307 lignin involves multi-step reactions that occur over a temperature range.²⁹

308 Other studies have used Near Infrared Spectroscopy (NIRS) to quantify lignin content
309 in similar samples. The calibration produced by⁴² was of poorer quality (R^2_{cv} 0.77; RPD 2.1)
310 than those developed in our work, while those reported by^{43,44} could be regarded as being of
311 similar or better quality (R^2_{cv} 0.94; RPD 4.0 and R^2_{cv} 0.95; RPD 4.4 respectively) than ours.
312 All of these studies used larger sample sets (n = 84-299). A Hi-Res TGA calibration model
313 created for lignin prediction in willow biomass²⁸ reported an R^2_c of 0.76, while lignin
314 calibrations developed by¹ for switchgrass using FTIR and Pyrolysis Molecular Beam Mass
315 Spectrometry (PyMBMS), reported statistics of R^2_{cv} 0.96 and R^2_{cv} 0.94 respectively. In
316 conclusion, this work has demonstrated that the three rapid analytical technologies

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

330 **ABBREVIATIONS USED**

331 ADF – Acid Detergent Fiber

332 AFBI – Agri-Food and Biosciences Institute

333 DM – Dry Matter

334 DTG – Derivative Thermogravimetry

335 FTIR – Fourier Transform Infrared

336 Hi-Res TGA – High Resolution Thermogravimetric Analysis

337 Max-Res TGA – Maximum Resolution Thermogravimetric Analysis

338 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^2_c - Coefficient of determination for calibration
- 348 R^2_{cv} - 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

355 **ACKNOWLEDGEMENT**

356 The authors would like to acknowledge the assistance of Prof Shekhar Sharma (AFBI,
357 retired) for his help with the multivariate analyses.

358

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477 **FUNDING**

478 The authors wish to thank the Department of Agriculture, Environment and Rural Affairs for
479 Northern Ireland for funding Evidence and Innovation Project 15-1-11 for the AFBI work
480 and AHDB Dairy Forages, Soils and Grasslands Research Partnership for funding the UoR
481 sheep digestibility work.

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 |
|---------------|--------------------------|-------|-------|-------|---------------------------|-------|-------|-------|
| | <u>UoR Diets (n=10)</u> | | | | <u>AFBI Diets (n=3)</u> | | | |
| 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 |
| | <u>UoR Faeces (n=10)</u> | | | | <u>AFBI Faeces (n=12)</u> | | | |
| 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 |
|-----------|--------------------------|------|------|------|---------------------------|------|------|------|
| | <u>UoR Diets (n=10)</u> | | | | <u>AFBI Diets (n=3)</u> | | | |
| 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 |
| | <u>UoR Faeces (n=10)</u> | | | | <u>AFBI Faeces (n=12)</u> | | | |
| 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 |
|-----------|--------------------------|------|------|------|---------------------------|------|------|------|
| | <u>UoR Diets (n=10)</u> | | | | <u>AFBI Diets (n=3)</u> | | | |
| 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 |
| | <u>UoR Faeces (n=10)</u> | | | | <u>AFBI Faeces (n=12)</u> | | | |
| 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^2_c , SEC) and Cross Validation (R^2_{cv} , 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^2_c | SEC | R^2_{cv} | 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

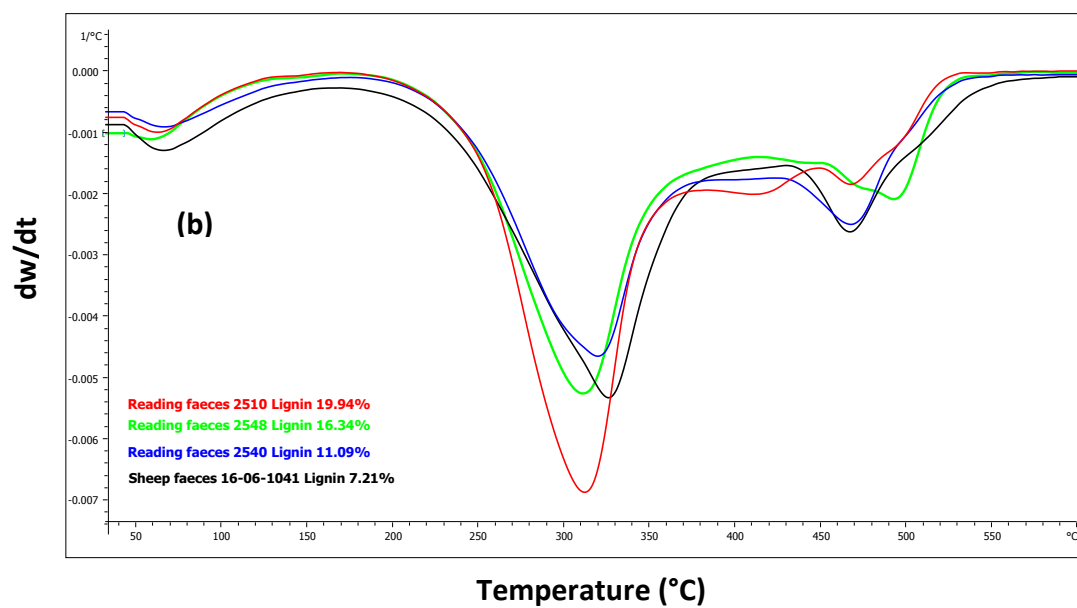
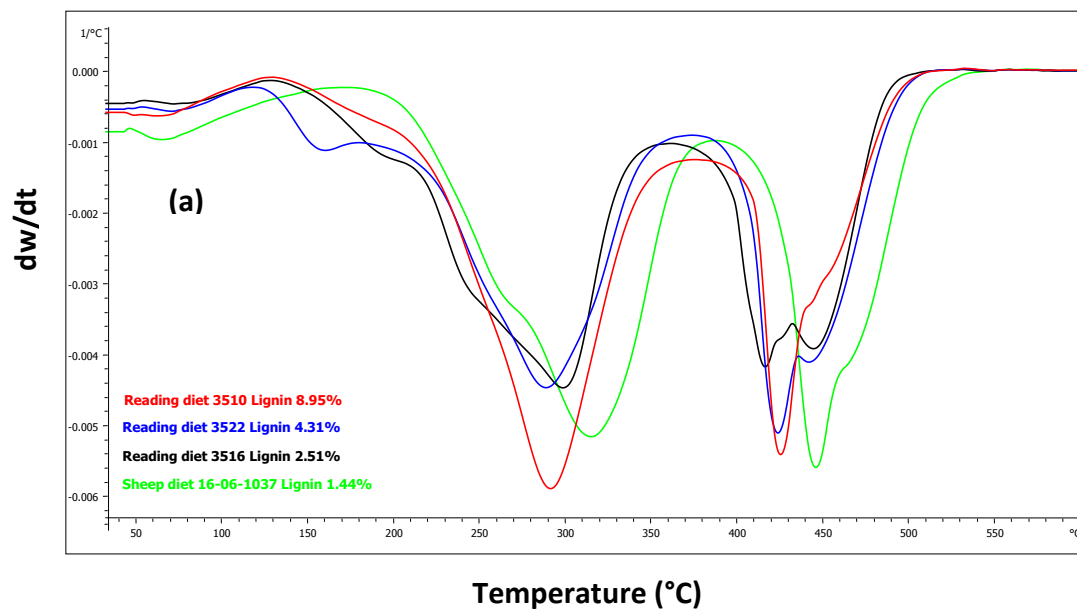


Figure 2. To be published in colour

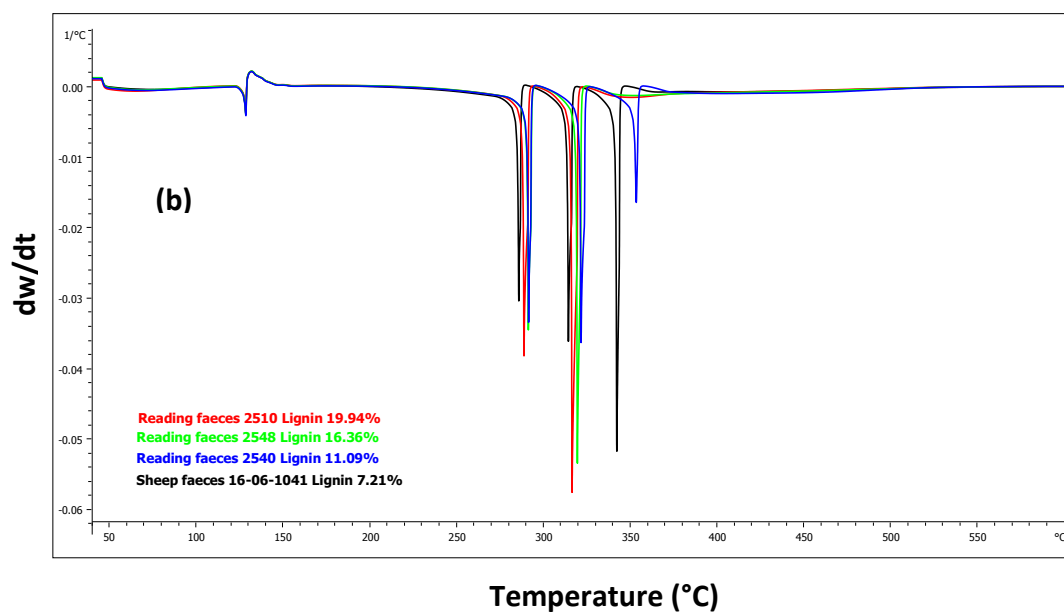
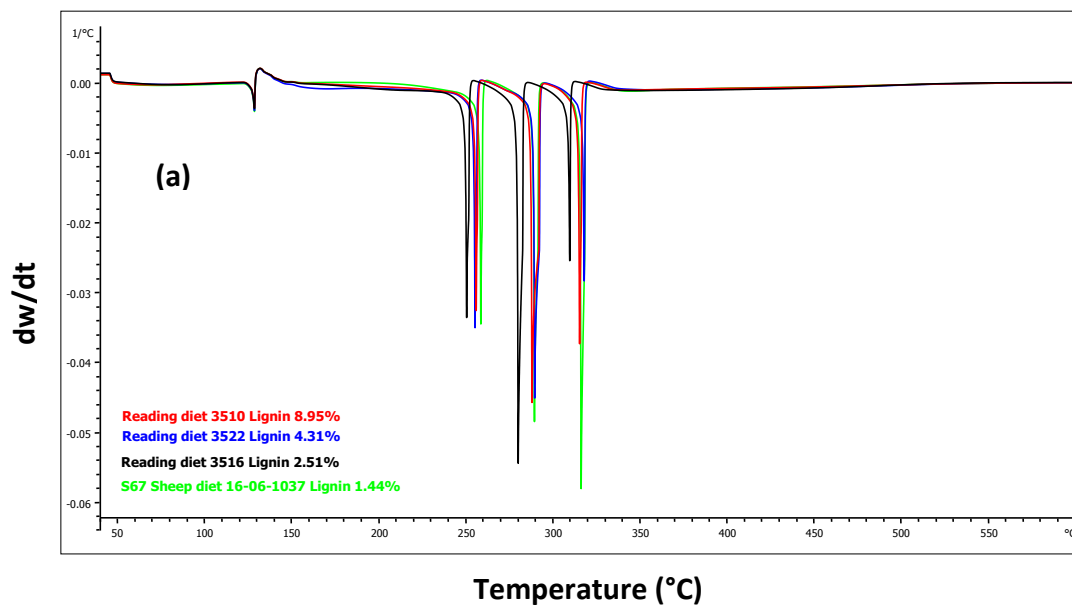


Figure 3. To be published in colour

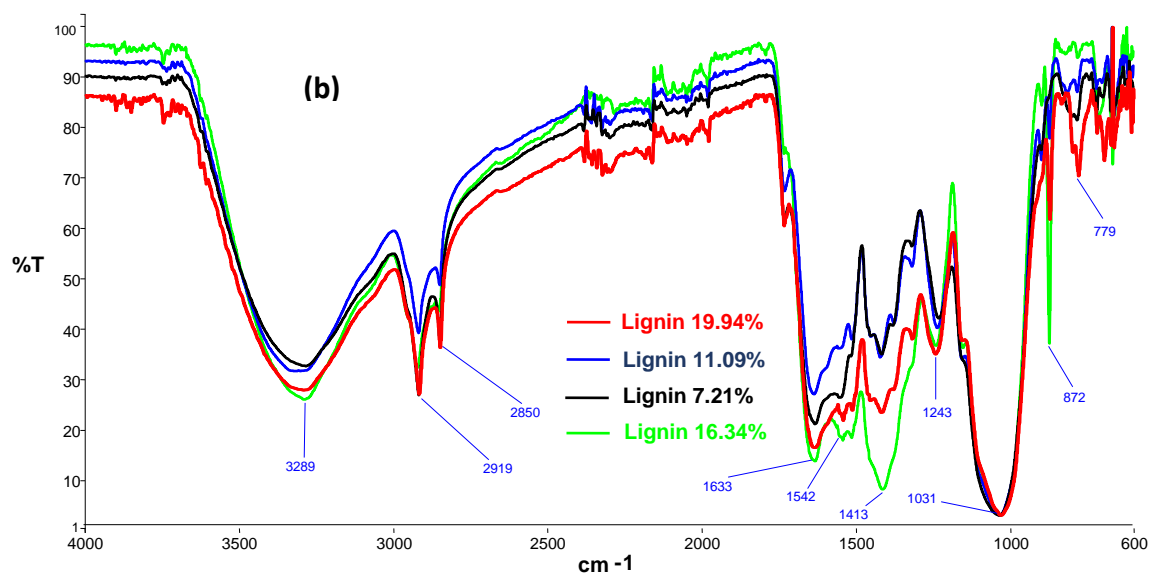
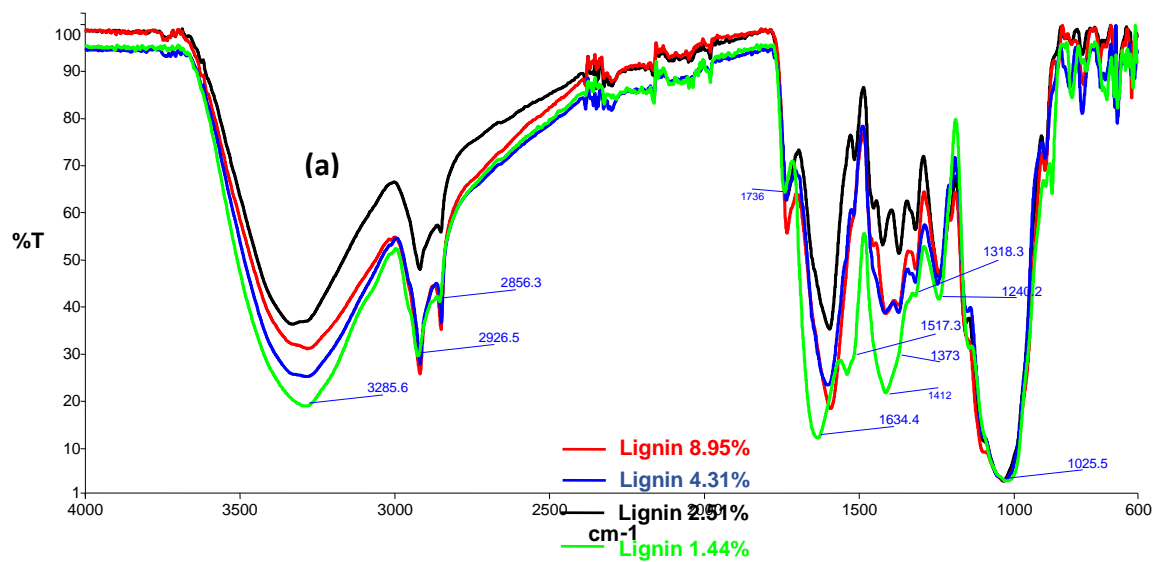
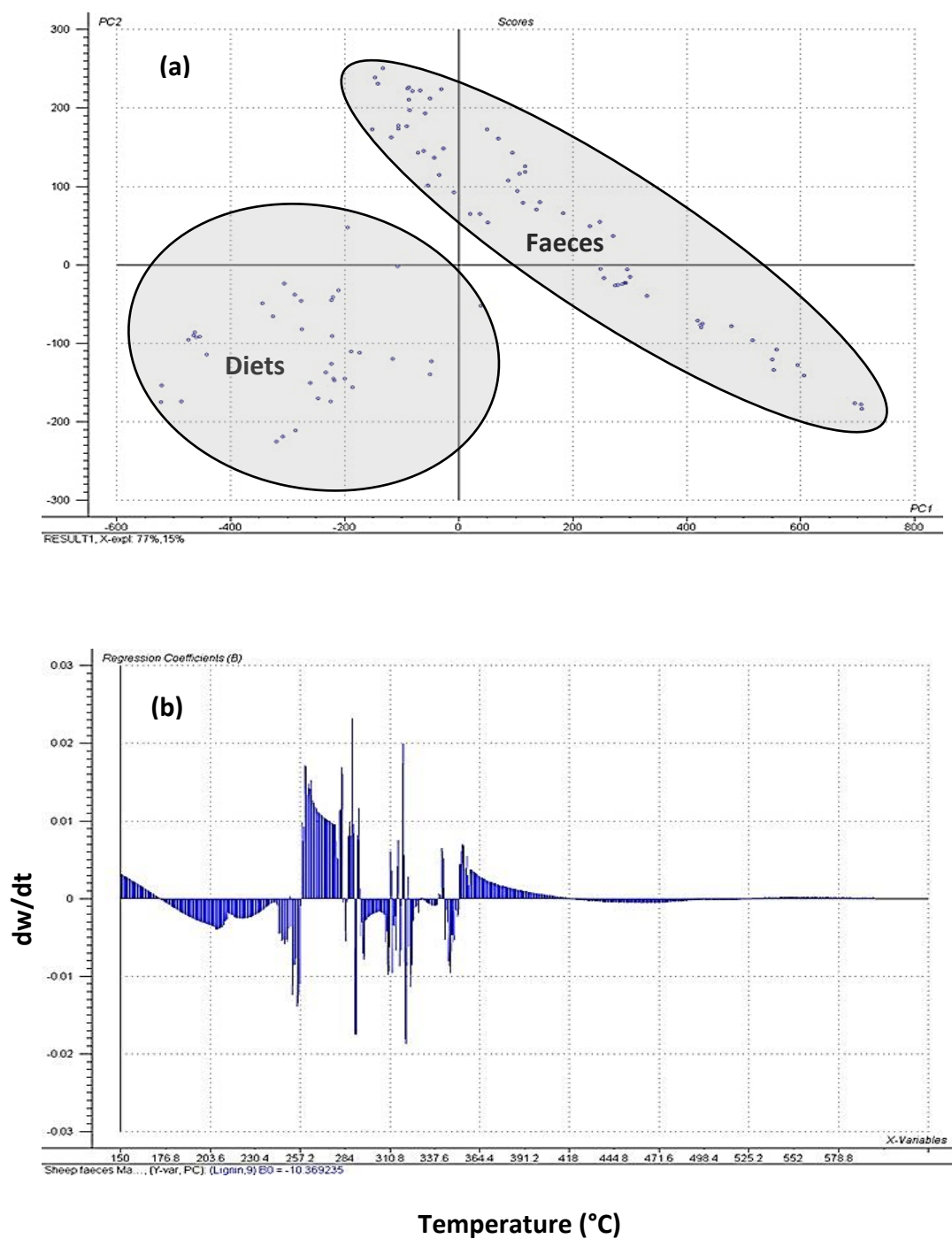
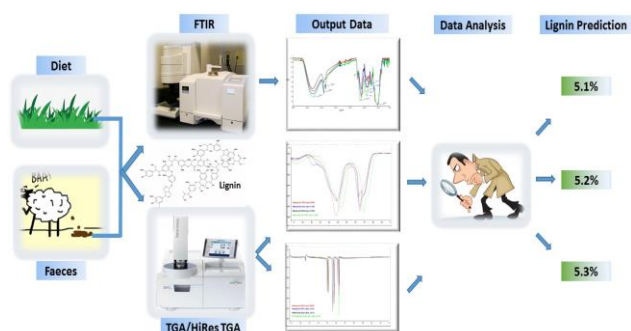


Figure 4.



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