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Near-Infrared Spectroscopy for Assessing the Chemical Composition and Fatty Acid Profile of the Total Mixed Rations of Dairy Buffaloes

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Featured Application: The role of fatty acids in the diets of animals, such as dairy buffaloes, is gaining increasing attention, particularly regarding polyunsaturated fatty acids (PUFAs) due to their impact on metabolic health, inflammation, and overall production efficiency. Monitoring the fatty acid composition in Total Mixed Rations (TMRs) through NIR spectroscopy is therefore strategic for ensuring the nutritional balance of the diet. The findings of this study not only reaffirm the well-established utility of NIR spectroscopy in assessing the chemical composition of animal rations but also establish a foundation for its application in evaluating fatty acid composition. This advancement enhances the potential for a comprehensive and integrated approach to feed analysis, enabling more effective on-farm verification and nutritional monitoring.

Abstract: Near-infrared spectroscopy (NIRS) is an efficient, non-destructive method for evaluating the chemical composition of various compounds. This study aimed to assess both the proximate composition, fibres, and fatty acid (FA) content of Total Mixed Rations (TMRs) in dairy buffalo nutrition. A total of 240 TMR samples were collected from ten dairy buffalo farms across four seasons to develop predictive models using Partial Least Squares Regression (PLSR). Calibration models for dry matter (DM), crude protein (CP), ether extract (EE), and starch demonstrated good predictive accuracy, with coefficients of determination in cross-validation (R^2_{cv}) around 0.90 and Residual Predictive Deviation (RPD_{cv}) values exceeding 3.0. Fatty acid models showed slightly lower R^2_{cv} values, ranging from 0.80 to 0.90. A good predictive performance was observed for linoleic acid (18:2 n-6) and α -linolenic acid (18:3 n-3), with RPD_p values above 3.0, indicating reliable predictions. The inclusion of omega-3-rich compounds in the diet provides significant benefits for both animal health and product quality, highlighting the importance of ration monitoring. The findings confirm that while NIRS is effective for assessing chemical composition, further refinement is needed to improve FA prediction accuracy. These results support the use of NIRS as a practical tool for nutritional monitoring in lactating buffaloes.

Keywords: near-infrared spectroscopy; fatty acids; total mixed ration; chemometric evaluation



Academic Editor: Franco Mutinelli

Received: 3 March 2025

Revised: 12 March 2025

Accepted: 13 March 2025

Published: 15 March 2025

Citation: Evangelista, C.; Contò, M.; Basiricò, L.; Bernabucci, U.; Failla, S. Near-Infrared Spectroscopy for Assessing the Chemical Composition and Fatty Acid Profile of the Total Mixed Rations of Dairy Buffaloes. *Appl. Sci.* **2025**, *15*, 3211. <https://doi.org/10.3390/app15063211>

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1. Introduction

The feeding of dairy ruminants, including buffalo, has become increasingly complex as it requires the precise incorporation of a wide variety of dietary components. This approach is aimed at meeting the metabolic demands of high-yielding animals while supporting optimal rumen function [1]. Buffaloes require a fibre-rich diet that varies with plant maturity stages and cultivation practices [2,3]. Carefully formulated rations are essential to prevent nutrient oversupply, reducing feed costs and minimising environmental waste [4]. To achieve nutritionally balanced diets, Total Mixed Rations (TMRs) are commonly used. These rations combine on-farm forages with concentrates and fatty compounds as well as vitamin and mineral supplements. The use of TMR facilitates the integration of critical nutrients, thereby supporting productivity, animal health, and sustainable production [3,5]. The rising demand for buffalo-derived products, such as mozzarella, has led to the increased mechanisation on buffalo farms, boosting farmer incomes and making TMRs the preferred feeding method. In this context, the effective management of dairy buffaloes now requires careful monitoring of rations and timely adjustments. Achieving optimal TMR composition not only requires a high-quality mixer-wagon but also continuous assessments of feed quality. This is particularly important in silage-based diets, where significant silage variability can impact the overall diet composition [6–8]. The evaluation of the chemical composition of ruminant diets, beyond merely providing balanced nutrition, is essential for enhancing the quality of animal-derived products. Among dietary components, starch and fibre fractions play a crucial role in modifying the nutritional and chemical quality of these products [9,10]. The fatty acid (FA) profile of TMR can also contribute to product quality [11]. Thus, despite the complexity in analysing the profile, there is a strong relationship between the quality of animal products and their diet. Nowadays, it is common practice to manipulate and enrich animal diets with particular FAs, aiming to deliver specific nutritional substances through the consumption of these products [12].

In this context, near-infrared spectroscopy (NIRS) emerges as an effective and versatile tool for modern feed analysis, providing rapid, non-destructive, and cost-effective evaluations [7,13]. The NIRS device enables the quick assessment of chemical composition in samples using the near-infrared spectrum, specifically at wavelengths from 900 nm to 2500 nm. It is commonly used to analyse chemical components in raw materials such as corn, soybean meals, and forages [14,15], as well as to evaluate blended raw materials in TMRs [16–18]. NIRS is also a valuable tool for studying the digestion process in livestock through the analysis of both feed and faecal samples [19–21].

Several NIR instruments with different features and performance levels have been employed to develop robust predictive models and to provide accessible and affordable options for on-site analysis [15,22]. Benchtop NIRS devices have long been fundamental in analytical contexts due to their high precision, stability, and ability to analyse a wide variety of samples with minimal interference [14]. Over the years, these instruments have undergone significant advancements in spectral resolution, data processing capabilities, and the development of sophisticated calibration models, establishing themselves as the benchmark for in-depth, highly detailed, and accurate analyses [16].

In NIR spectrometry, spectral bands correspond to specific molecular vibrations of chemical bonds (e.g., C-H, N-H, and O-H), allowing for the identification and quantification of various components [23]. The NIR spectrum comprises fundamental, overtone (first, second, and third harmonics), and combination bands. Due to significant spectral overlap, NIR spectra are often complex, with broad peaks that can obscure finer details, especially in biomolecular samples where multiple bond types contribute to the overall signal [24]. To interpret the complexity of these unresolved spectral bands in NIR spectra, chemomet-

ric analyses are applied to improve peak resolution and minimise spectral interference from overlapping bands [25,26]. The large number of spectral variables in NIR data can introduce significant noise and interference, potentially compromising the reliability of predictive models. However, Partial Least Squares Regression (PLSR) mitigates the noise by reducing dimensionality through latent variables (LVs), which minimise spectral overlap and improve model accuracy [27]. However, few studies have validated NIRS for determining the FA composition of forages, and its application in analysing the FA within complex matrices like TMRs is rare. Foster et al. [28] reported validation results with R^2 values ranging from 0.86 to 0.97 for FAs in different types of forage. Other authors [29] found lower R^2 values, ranging from 0.64 to 0.89. Recently, Arslan et al. [30] evaluated the FA composition in different types of flaxseeds, obtaining R^2 values between 0.71 and 0.85. However, they highlighted the excellent performance of Fourier Transform NIR (FT-NIR) in evaluating proteins ($R^2 = 0.87$) and Neutral Detergent Fibre (aNDF) ($R^2 = 0.90$). Although fat constitutes a relatively minor component of the ration, comprising approximately 2–5% of dry matter, it plays a crucial role in several physiological processes. It facilitates the absorption of essential FAs, helps mitigate inflammatory processes (e.g., through omega-3 FA intake), enhances the absorption and utilisation of lipophilic compounds such as vitamins and polyphenols, and modulates ruminal activity [31]. Additionally, dietary fats originate from various sources, including forages, concentrates, and vegetable oils, adding to the compositional variability. The separation of fibrous, starchy, and oily components can often compromise measurement accuracy, making representative spectral determinations more challenging.

This study aims to address these challenges by developing predictive models to estimate the chemical composition, particularly the FA profile, of TMRs used in ten buffalo dairy farms across four seasons. Specifically, it evaluates the effectiveness of NIR spectrometry in constructing these predictive models.

2. Materials and Methods

2.1. Study Design

The TMR samples used in this study were obtained from the study by Evangelista et al. [3], which monitored ten dairy buffalo farms in the Lazio region supplying milk for buffalo mozzarella production over an entire year, collecting a total of 240 TMR samples.

The rations in the ten farms were adjusted monthly based on the availability of forage in each farm. The rations across the farms were monitored for one year, during which a total of 240 samples were collected.

Each sample, weighing approximately 1 kg, was taken immediately after the morning feeding, stored under refrigerated conditions (4 °C) for transport, and briefly held before being dried.

The principal components of the TMR consisted of corn silage and/or grass silage, with a wide variation among farms ranging from 24.5% to 73.6%. In general, farms provided more silage during the autumn and winter months. The other fibrous component of the ration was hay, contributing between 3.7% and 32.7%, of which approximately 30% was alfalfa hay. Five farms provided fresh grass during the spring period, reaching values of up to 30% of the ration.

Fibre supplementation with straw was used by three farms, with an average contribution of 5%. Another three farms supplemented the ration with an average of 20% brewers' grains. Highly energetic concentrates, such as corn or barley meals, were used by all farms and ranged from 2.5% to 21%, while highly concentrated protein feeds, such as soybean or faba bean meal, varied among farms throughout the year from 1.5% to 12%.

of the ration. Three farms provided an average of 1.6% Linomix (nutritional supplement based on extruded linseeds), while one farm supplemented the ration with 5% cottonseed.

Due to their number and wide variability, the collected samples provided a valid dataset for NIR analysis.

Spectrometric measurements were conducted with the benchtop NIRFlex 500 (BÜCHI, Flawil, Switzerland). The measurements were performed after sample drying, which was carried out in the laboratory and processed in parallel with the chemical analyses.

2.2. Chemical Composition and Fatty Acid Analysis of Total Mixed Ration

The dry matter (DM) of TMR samples was measured after oven drying at 65 °C to constant weight and expressed as a % of the feed. The dry samples were ground through a mill (Retsch Müller, Haan, Germany) to pass a 1 mm screen and used for chemical analysis and spectrometric determination. Samples were analysed for ash, crude protein (CP), ether extract (EE) [32], and total starch using a K-TSTA assay kit (Megazyme International, Bray, Ireland). Neutral Detergent Fibre (aNDF), Acid Detergent Fibre (ADF), and Acid Detergent Lignin (ADL) were analysed using an Ankom200 Fiber Analyzer (Ankom Technology, Macedon, NY, USA) according to Van Soest et al. [33]. The crude fibre (CF) content was determined with the same instrument, according to the Weende method [32]. Chemical data are reported as percentages of DM.

The extraction of FAs followed the method of Folch et al. [34] using a chloroform/methanol mixture (2:1, *v/v*). Fat dissolved in hexane was methylated with methanolic KOH, and a 1 µL sample was injected into a gas chromatograph with a flame ionisation detector (GC-FID) (Agilent Technologies, Santa Clara, CA, USA), equipped with a CP-Sil88 capillary column (100 m, 0.25 mm, 0.20 µm, 100% cyanopropyl, Agilent Technologies). The fatty acid 19:0 (Sigma-Aldrich Merck, Darmstadt, Germany) was used as the internal standard. GC-FID conditions were as follows: splitless inlet at 250 °C; FID at 250 °C; helium carrier gas at 1.2 mL/min. The oven temperature programme began at 60 °C (held for 4 min), then ramped up by 13 °C/min to 175 °C (held for 27 min), followed by an increase of 4 °C/min to 215 °C (held for 25 min), and a final increase by 2 °C/min to 220 °C (held for 5 min) as previously reported by Contò et al. [35]. Fatty acid peaks were identified by comparing them to peaks in a Supelco mix 37 standard (Sigma-Aldrich Merck, Darmstadt, Germany); which includes the main fatty acids typically found in the TMRs such as myristic acid (14:0), palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1 *cis*-9), vaccenic acid (18:1 *cis*-11), linoleic acid (18:2 *n*-6), γ -linolenic acid (18:3 *n*-6), and α -linolenic acid (18:3 *n*-3). Fatty acid methyl esters (FAMES) were expressed as a percentage of the total FAME content. The total amounts of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs) were also calculated.

2.3. Collection of NIR Spectra

Spectra were acquired from dried samples using the NIRFlex 500, and more specific details are provided in Table 1.

The minced, dried sample was placed into a cylindrical quartz glass cup with a diameter of 25 mm and a height of 10 mm. To ensure better spectral averaging across the sample surface, a sample rotation accessory was employed. For each spectrum, 32 scans were taken, and each sample was scanned three times, using, as the final data, their mean. Spectral acquisition was conducted at room temperature (approximately 20–21 °C).

The benchtop spectrometer collected 1501 data points within the standard spectral range of 10,000 to 4000 cm^{−1} (1000–2500 nm).

Table 1. Technical specifications of the NIRS instrument used in the study: NIRFlex 500 BÜCHI (benchtop).

Type	NIRFlex 500 BÜCHI
Sensor	Extended range InGaAs (temperature controlled)
Spectrum range	1000–2500 nm
Numerical resolution	4 cm ^{−1} (with boxcar apodisation)
Average optical resolution	HWHN 3.25 nm
Supply	100–230 VAC ± 10%, 50/60 Hz, 350 W
Type of measurements	Reflectance/transmittance
	Measurement cells (NIRFlex Solids)
Measurement geometry	FT-NIR
	Internal and automatic

Spectral data were initially recorded as reflectance (R) and subsequently converted to absorbance ($\log 1/R$). All chemometric processing and analyses were performed using Unscrambler X, version 10.3 (CAMO Software, Oslo, Norway). Spectral data were used as the independent variable to predict the concentrations of chemical constituents, and of FAs which were designated as the dependent variables.

2.4. Division of Dataset and Spectra Preprocessing

The dataset obtained from 10 farms was partitioned using data recorded from 7 buffalo farms for model training, while data from 3 randomly selected farms were used as an independent dataset. This resulted in a calibration dataset comprising 168 samples and a validation dataset consisting of 72 samples. This partitioning ensures that the two datasets remain independent.

A basic statistical analysis was conducted on these datasets for each dependent variable. The mean, standard deviation (S.D.), minimum and maximum range, and coefficient of variation (C.V.) were calculated.

Spectral data were preprocessed using techniques such as data normalisation and baseline correction. To optimise the model, scattering correction methods such as Standard Normal Variate (SNV) and detrending were used. Additionally, a derivative as a mathematical transformation was applied to enhance the accuracy of the Partial Least Squares Regression (PLSR) model [36–38].

2.5. Partial Least Squares Regression (PLSR) Models

Partial Least Squares Regression was chosen for its ability to handle highly collinear spectral data by compressing multiple data points into fewer representative factors, known as LVs. These LVs capture the main variance in the data while minimising redundancy, allowing for effective model calibration. Calibration equations were specifically developed for the chemical constituent and FA fractions.

The calibration dataset was used to build the PLSR model by applying five-fold cross-validation, a method particularly well suited for the relatively small dataset size and the presence of repeated measurements. This approach divides the calibration data into five subsets, with four subsets used for model training and the remaining subset for validation in each iteration. This method ensures that all samples are included in cross-validation phases [37].

The optimal calibration models were selected based on key performance metrics reflecting the robustness of each model. Subsequently, the PLSR model was used in the external validation dataset to estimate the performances of the prediction models.

This study considered several goodness-of-fit statistics, including the coefficients of determination for calibration, cross-validation, and prediction (R^2_c , R^2_{cv} , and R^2_p ,

respectively). The standard errors of calibration (SECs), cross-validation (SECVs), and prediction (SEPs) were also evaluated, with the ideal being as low as possible to indicate the high predictive accuracy of the model. The Residual Predictive Deviation (RPD = SD/SECV or SEP) was also calculated, with its classifications outlined in Table 2. In addition, the range error ratio (RER = Range Of Reference Values/SECV or SEP) was calculated [37]. A high R^2 , an RPD value greater than 3, and an RER value greater than 10 suggested that the model retains good predictive capabilities across different datasets [39].

Table 2. Classification and success levels based on R^2 , RPD, and RER as per references [39,40].

R^2	RPD	RER	Classification	Application
<0.80	<2.0	<7	Very poor	Not recommended
	2.0 to 2.5		Poor	Rough screening
0.80 to 0.90	2.5 to 3.0	<10	Fair	Screening
	3.0 to 3.5	>10	Good	Quality control
0.90 to 0.95	3.5 to 4.0		Very good	Process control
>0.95	>4.0	>20	Excellent	Any application

R^2 = coefficients of determination; RPD = Residual Predictive Deviation (S.D./SEC); RER = Range of Reference Values (S.D./range).

3. Results and Discussion

3.1. Sample Composition

Nine chemical parameters, nine individual FAs, and three FA classes were analysed for each TMR sample. The results of the descriptive analysis (mean, range, standard deviation, and coefficient of variation) are presented in Table 3 for the TMR chemical composition and in Table 4 for the FA content, and they are presented separately for the calibration and validation sets.

Table 3. Descriptive statistics of the chemical composition (% of DM) of Total Mixed Rations (TMRs) from both the calibration (n = 168) and validation (n = 72) sets.

Calibration Set (168)					Independent Set for Validation (72)			
%	Mean	Range	S.D.	C.V.	Mean	Range	S.D.	C.V.
DM *	52.33	25.48	5.65	10.80	52.34	22.04	5.73	10.95
Ash	6.76	3.69	0.79	11.75	6.83	3.81	0.85	12.40
CP	12.08	9.31	1.58	13.09	12.59	9.57	1.82	14.45
EE	2.50	2.45	0.39	15.64	2.45	2.03	0.42	17.10
CF	28.96	24.54	5.26	18.16	30.08	21.61	4.97	16.52
aNDF	44.72	25.72	5.01	11.21	46.56	23.49	5.16	11.08
ADF	30.40	15.63	3.35	11.02	31.38	17.22	3.70	11.78
ADL	6.45	5.95	1.18	18.31	6.97	5.82	1.20	17.26
Starch	14.50	12.85	2.73	18.80	13.18	12.05	2.65	20.08

S.D. = standard deviation; C.V. = coefficient of variation (S.D./Mean $\times$ 100); range (max value–minor value).

* = expressed as % of TMR; DM = dry matter; CP = crude protein; EE = ether extract; CF = crude fibre; aNDF = Neutral Detergent fibre; ADF = Acid Detergent Fibre; ADL = Acid Detergent Lignin.

Buffalo rations in this study were inherently more fibrous and had a distinct nutrient profile compared to those formulated for cattle or other buffalo rations [41,42]. A wide variability was observed in the chemical composition of the TMRs in both the calibration and validation sets (Table 3), particularly for starch, crude fibre, and ADL with C.V. > 18%, while in the validation set, only starch exceeded this value (C.V. = 20.1%). Conversely, the lowest variability (<13%) was noted for DM, Ash, aNDF, and ADF in both the calibration and validation sets.

Table 4. Descriptive statistics of fatty acids (% of total FAMES) in Total Mixed Rations (TMRs) from both the calibration (n = 168) and validation (n = 72) sets.

Calibration Set (168)					Independent Set for Validation (72)			
%	Mean	Range	S.D.	C.V.	Mean	Range	S.D.	C.V.
14:0	0.47	0.52	0.10	20.64	0.50	0.48	0.10	20.80
16:0	17.36	8.89	1.83	10.56	18.05	7.50	1.73	9.57
16:1	0.46	0.70	0.12	25.22	0.46	0.67	0.10	21.09
18:0	3.12	3.76	0.63	20.26	3.08	2.98	0.61	19.77
18:1 cis-9	20.89	8.69	1.81	8.65	20.53	8.55	1.61	7.82
18:1 cis-11	1.03	0.56	0.10	9.81	1.05	0.48	0.10	8.95
18:2 n-6	42.54	21.57	3.62	8.04	43.25	17.88	3.73	8.62
18:3 n-6	1.78	4.17	0.87	48.99	2.01	3.29	0.79	42.49
18:3 n-3	9.77	13.98	3.59	36.77	8.64	13.77	3.96	45.84
SFA	21.81	7.91	1.99	9.11	22.21	7.81	1.81	8.13
MUFA	22.89	7.52	1.67	7.30	22.48	7.87	1.66	7.36
PUFA	55.19	12.16	2.28	4.14	54.89	9.91	1.98	3.61

S.D. = standard deviation; C.V. = coefficient of variation; SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid.

The FAs present in the TMRs are derived from both concentrate sources (corn, soya, barley, and field bean), fodder sources (green forage, hays, and silage), and some supplements, such as Linomix, providing significant sources of 18:3 n-3. As a result, there is considerable variability in the FA composition of the TMRs (Table 4). For 18:3 n-3 and 18:3 n-6, the C.V. exceeds 35%. In contrast, the lowest variability (<10%) was observed for 18:1 cis-9 and 18:1 cis-11, 18:2 n-6, for the FA classes (SFA, MUFA, and PUFA), and for 16:0 in the validation set.

Although several studies have explored various chemical characteristics of TMRs for dairy buffalo, few authors have specifically examined the FA profiles. This makes it difficult to directly compare our findings with previous research. Existing studies have primarily focused on grazing systems, reporting low fat content [28,43], or on individual types of concentrates. In contrast, this study examined TMRs, a mixture of forages and concentrates that collectively contribute to the higher fat content observed in the diet.

In general, a robust calibration set should have exhibited sufficient variability to enhance the model's reliability, generalisability, and resistance to overfitting [37,44,45]. In this study, the inclusion of data from 10 dairy buffalo farms checked during different seasons of the year ensured high variability in terms of TMR composition.

3.2. Spectral Characteristics

The NIR spectral characteristics of dried TMR samples exhibit distinct peaks, as shown in Figure 1.

The samples, even if dried, maintained water absorption bands due to O-H groups, around 1400 nm and 1900 nm, producing sharper, more defined peaks, and offering enhanced resolution for organic compounds such as proteins, fats, fibres, and FAs.

The C-H absorption bands associated with some FAs are detectable in the second and third harmonic overtones. As reported by Giarretta et al. [46], FAs absorb within the C-H combination stretching region (2200–2500 nm).

The harmonics of NIR refer to absorption phenomena within the NIR range (780–2500 nm), driven by molecular vibrations in functional groups containing bonds such as C-H, O-H, and N-H [47]. Proteins absorb primarily due to N-H and C-H bonds, with main absorption bands around 2050–2250 nm (amino groups) and 1600–1700 nm (secondary bands). Fats and FAs show absorption related to C-H groups, with key bands at

1700–1800 nm (stretching of CH_2 and CH_3) and 2300–2400 nm (specific to saturated/unsaturated fats). Fibres like cellulose and hemicellulose absorb in the 2100–2200 nm and 1700–1800 nm regions, which are associated with O-H and C-H bonds in complex carbohydrates [48].

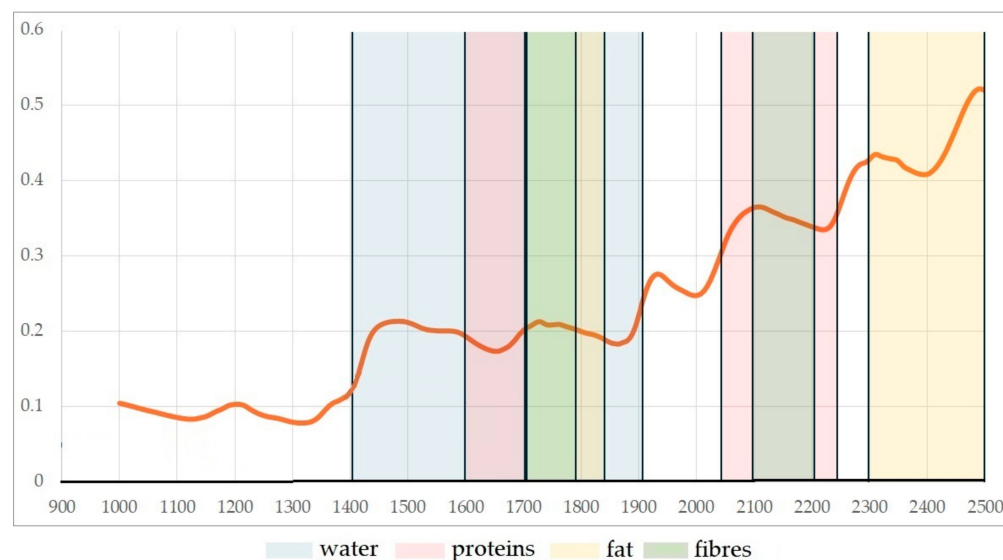


Figure 1. NIR spectra from NIRFlex 500 with the typical band of chemical compound absorption. Shaded areas highlight characteristic absorption bands of specific chemical components: light blue for water, pink for proteins, yellow for fats, and green for fibers.

In the near-infrared spectrum, overtones and combination bands are associated with specific spectral regions and enable the detection of various compounds. The first overtone, typically observed between 1450 nm and 1900 nm, is dominated by C–H, N–H, and O–H vibrations and is particularly useful for identifying water, proteins, and fats. The second overtone, found in the range of 900 nm to 1300 nm, involves the same types of bonds but exhibits lower intensity. This region is commonly employed for the analysis of fats, carbohydrates, and proteins. The third overtone, located between 750 nm and 900 nm, is characterised by very low intensity, making its detection reliant on highly sensitive instruments. Consequently, it is less frequently used in practical applications. Combination bands, generally observed in the spectral range of 1900 nm to 2500 nm, are more intense than higher-order overtones and are particularly important for analysing complex chemical compositions such as proteins, starches, and FAs [24]. The plots of the variables contributing to the PLSR models for some compounds, for example, are provided in the Supplementary Materials (Figure S1).

3.3. Pre-Treatment of NIR Spectra

The pre-treatment of NIR spectra is important because raw spectral data often contain noise, baseline shifts, and scattering effects, which may arise from the physical characteristics of TMR samples or from instrumental factors.

These interferences can obscure some peaks and, consequently, chemical information in the spectra, making it difficult to develop accurate predictive models [26,28]. For this purpose, preprocessing techniques like SNV are employed to mitigate scattering effects [26,49]. In addition, NIR spectra typically produce low-resolution and poorly defined signals. Therefore, for some chemical models, a mathematical treatment—such as the first derivative using the Savitzky–Golay algorithm—was applied to transform subtle oscillations into discernible peaks. The derivative applied to the preprocessing data was coded with the sequence 1-2-2-1, where the first value corresponds to the derivative order,

the second denotes the interval used for its calculation, the third serves as a smoothing factor representing the number of data points included in a running average, and the fourth indicates the secondary smoothing. Various preprocessing techniques, including the second derivative, were tested; however, they resulted in minimal improvements in cross-validation performance. Therefore, a limited number of preprocessing steps were applied to prevent overfitting.

The pre-treated spectra were subsequently used with PLSR analysis to develop models that maximised R^2 and minimised the SEC for each analyte studied. The most frequently applied spectral pre-treatments in our data are shown in Figure 2.

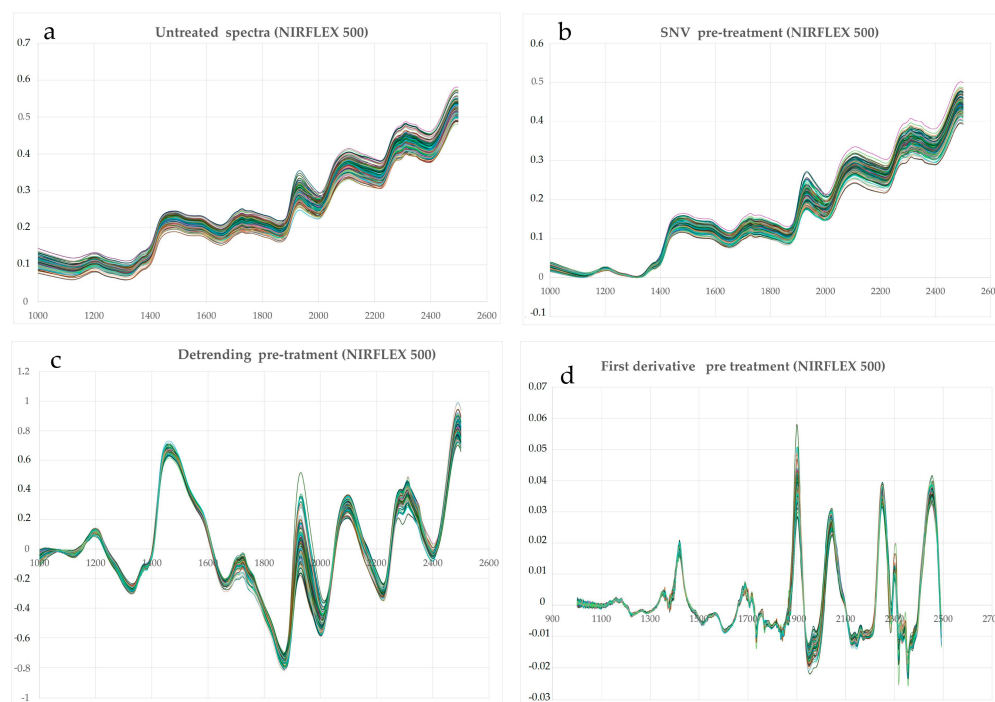


Figure 2. The trends in the spectral data with the pre-treatment applied. Note: (a) = untreated spectra; (b) = SNV; (c) = detrending; and (d) = first derivative (1-2-2-1).

3.4. Prediction Models

The PLSR models were evaluated to determine their reliability and practical usefulness for each chemical compound and FA.

Calibration, cross-validation, and validation statistics for the PLSR models related to proximate composition, fibre constituents and FAs are presented in the following subsections. In the same tables, the outlier and LV are reported for each compound. Additionally, in the Supplementary Materials, the best PLSR models for calibration and cross-validation analysis are provided in Figure S2a–d, while Figure S3 presents the plot of prediction analysis on an independent dataset.

The outliers in PLSR analysis were identified and removed using statistical tools such as Leverage and Q residuals (or Squared Prediction Errors). Leverage measures the distance of a sample from the average in the predictor space.

Q residuals (or Squared Prediction Errors) are statistical measures used in multivariate analysis, particularly in PLSR. Mathematically, Q residuals are defined as the sum of the squared differences between observed values and those estimated by the model. High Q residuals suggest that the sample contains variability not captured by the model, whether during calibration or validation. When both Leverage and Q residuals are significantly high, it is likely that the sample is an outlier. Removing these outliers can improve the model's robustness and reduce SEC.

3.4.1. Prediction Model for Chemical Analysis

Among the PLSR models obtained with NIR spectra, no outliers were identified for DM, CP, and starch, while the other parameters had outlier values ranging from 2 to 5 in the calibration set. The outliers in the validation set did not exceed three units for all the models analysed, with EE, CF, and starch having no outliers identified.

In the same tables (Table 5), the latent variables of the calibration model are reported, ranging from a minimum of 4 to a maximum of 7.

Table 5. Calibration, cross-validation (cv), and prediction (p) statistics for the chemical composition (% of DM) of Total Mixed Rations (TMRs) from both the calibration and validation set.

%	LV	OUT	Calibration Set				Independent Set for Validation		
			R ² c	SEC	R ² cv	SECV	OUT	R ² p	SEP
DM *	7	0	0.93	1.22	0.92	1.32	1	0.89	1.41
Ash	5	5	0.87	0.25	0.86	0.26	1	0.85	0.29
CP	4	0	0.91	0.45	0.90	0.47	2	0.88	0.58
EE	6	2	0.90	0.12	0.89	0.13	0	0.87	0.15
CF	7	4	0.87	1.55	0.85	1.68	0	0.84	1.77
aNDF	7	5	0.90	1.41	0.88	1.55	1	0.81	1.71
ADF	6	4	0.88	1.07	0.87	1.12	1	0.85	1.36
ADL	5	2	0.83	0.39	0.82	0.42	3	0.81	0.43
Starch	7	0	0.92	0.74	0.91	0.79	0	0.88	0.82

* = expressed as % of TMR. LV = latent variable; OUT = outlier; R²c = coefficient of determination on calibration set; SEC = standard error on calibration set; R²cv = cross-validation coefficient of determination on calibration set; SECV = cross-validation standard error on calibration set; R²p = prediction coefficient of determination on validation set; SEP = prediction standard error on validation set; DM = dry matter; CP = crude protein; EE = ether extract; CF = crude fibre; aNDF = Neutral Detergent Fibre; ADF = Acid Detergent Fibre; ADL = Acid Detergent Lignin.

The optimal number of LVs was selected based on the explained variance plateau criterion, ensuring that additional LVs were included only when they significantly improved model performance. Furthermore, to prevent overfitting, we set a maximum limit of seven LVs.

Considering the chemical composition, good calibration models (R²c > 0.90) were obtained for DM, CP, EE, aNDF, and starch. However, the R² for each parameter in calibration, cross-validation, and prediction did not fall below 0.8.

In the five-fold cross-validation, the R² value decreased, as expected, for all the chemical components considered, achieving an R²cv > 0.90 only for DM, CP, and starch. For all parameters evaluated, the SECV was slightly higher than the SEC, but it did not exceed 10 percentage points (p.p.). When comparing SECV with SEP, the increase ranged from a minimum of 0.15 p.p. to a maximum of 1.77 p.p. for CF.

To assess model performance based on the RPDcv value (Table 6), following the guidelines provided by [39,40] and as outlined in Table 2, no parameters had an RPDcv lower than 2, thereby excluding any models classified as poor and not recommended for use. The RPDcv values exceeded the threshold of 2.5 for almost all parameters, with DM surpassing the threshold of 4. Notably, all parameters had an RERcv greater than 10.

The external validation, using the validation set, is considered essential for evaluating the applicability of the model to independent datasets. For this prediction analysis, the R² of the considered chemical parameters ranged from 0.81 to 0.89, demonstrating “fair” performance [39].

In the prediction phase, due to the increase in SEP compared to SECV, the RPDp values were lower. However, DM maintained a value above 4, despite being slightly lower than in cross-validation. The remaining parameters ranged from a minimum of 2.7 to a maximum

of 3.22. In prediction, DM, CP, aNDF, and starch maintained performances classified as good, while the remaining parameters were rated as fair. No chemical parameter showed a predictive model classified as poor.

Table 6. Residual Predictive Deviation (RPD) and Range of Reference Values (RER) on cross-validation (cv) and prediction (p) for chemical composition (% g of DM) of Total Mixed Rations (TMRs).

%	Calibration Set		Independent Set for Validation	
	RPD _{cv}	RER _{cv}	RPD _p	RER _p
DM *	4.29	19.36	4.06	15.62
Ash	3.04	14.14	2.95	13.28
CP	3.32	19.54	3.12	16.42
EE	2.92	18.28	2.70	13.10
CF	3.14	14.64	2.80	12.18
aNDF	3.22	16.55	3.01	13.70
ADF	2.98	13.89	2.71	12.62
ADL	2.83	14.27	2.80	13.57
Starch	3.45	16.25	3.22	14.68

* = expressed as % of TMR; RPD_{cv} = SD/SEC_v; RPD_p = SD/SEP; RER_{cv} = range (max-min value)/SEC_v; RER_p = range (max-min value)/SEP; DM = dry matter; CP = crude protein; EE = ether extract; CF = crude fibre; aNDF = Neutral Detergent Fibre; ADF = Acid Detergent Fibre; ADL = Acid Detergent Lignin.

For all tested models, the RER remained consistently above 10, ranging from 13.89 for ADF to 19.54 for CP in cross-validation and from 12.18 for CF to 16.42 for CP in prediction.

NIRS studies related to TMRs are relatively limited compared to those conducted on forages and concentrates considered individually, with a notable lack of research focused on TMRs for dairy buffaloes. The study by Buonaiuto et al. [8], conducted on rations for cows producing milk for Parmigiano Reggiano cheese, could serve as a useful point of comparison, as the rations used in this experiment were significantly richer in fibre than those typically used for dairy cows. However, it is important to note that the rations for Parmigiano Reggiano do not include silage, while silage is commonly used in buffalo diets, including the rations considered in this study. Buonaiuto et al. [8] achieved good results for CP, starch, aNDF, and ADF ($R^2_c > 0.80$), with lower values for ADL ($R^2_c = 0.76$) and ash ($R^2_c = 0.57$). The RPD values for these components were greater than 2.20, while they were lower for ADL and ash (< 1.90). They reported RMSE values for CP, ADL, and ash lower than 1, while for the other parameters, the RMSE was found to be greater than 2, which is higher compared to our data.

Pérez-Marín et al. [50] developed calibration models for 394 TMR samples for dairy cows, obtaining coefficients of determination (R^2_c) greater than 0.80, except for starch, which had an R^2_c of 0.75. The SEC ranged from 0.71 to 1.25, and the RPD values ranged from 2.31 to 5.76. The best calibration results were achieved for DM. These models were developed using the Foss NIRSystems 6500 instruments (1100–2498 nm). Excellent calibration results were also published by Pereira-Crespo et al. [18] in a study involving 125 samples of TMRs for dairy cows. They achieved high coefficients of determination for calibration ($R^2_c > 0.98$) with very low errors (SEC < 0.67) and cross-validation RPD values greater than 3, indicating excellent model capability according to the discussed metrics.

3.4.2. Prediction Model for Fatty Acids

For FAs, the outliers ranged from 0 to 4 for the calibration and validation sets, and the LVs used for each model ranged from 4 to 7 (Table 7).

Considering the PLSR models for FAs, the NIRflex system achieved good R^2 values during calibration for all FAs. The 18:3 n-6 showed $R^2_c = 0.91$, while 18:1 cis-11 and the SFA and PUFA classes had R^2_c values < 0.85 . Unfortunately, in cross-validation using the

five-fold approach, the R^2_{cv} values slightly decreased, and the SECV increased compared to the SEC. Applying the PLSR models to the validation set, the R^2 for 18:3 n-6 was confirmed at 0.90. Lower predictive performance, with $R^2 < 0.80$, was recorded for 18:1 cis-11 and SFAs. The other FAs had R^2 values ranging from 0.80 to 0.84. When comparing SEP to SECV, lower values were observed for 16:1, 18:3 n-6, and the PUFA class. This occurred because the validation set exhibited a lower S.D. for certain FAs compared to the data in the calibration set (Table 4). However, SEP never exceeded 1.2.

Table 7. Calibration, cross-validation (cv), and prediction (p) statistics for the fatty acid composition (% of total FAMES) of Total Mixed Rations (TMRs) from both the calibration and validation set.

Calibration Set							Independent Set for Validation		
%	LV	OUT	R ² c	SEC	R ² cv	SECV	OUT	R ² p	SEP
14:0	4	0	0.86	0.03	0.84	0.03	0	0.83	0.04
16:0	7	4	0.85	0.60	0.83	0.65	3	0.82	0.65
16:1	6	2	0.88	0.04	0.86	0.04	2	0.84	0.04
18:0	7	3	0.87	0.17	0.85	0.18	2	0.83	0.24
18:1 cis-9	7	3	0.87	0.55	0.85	0.59	2	0.84	0.63
18:1 cis-11	7	3	0.84	0.03	0.81	0.03	1	0.78	0.04
18:2 n-6	7	2	0.88	1.10	0.86	1.20	1	0.84	1.21
18:3 n-6	6	0	0.91	0.21	0.90	0.23	0	0.90	0.21
18:3 n-3	7	3	0.86	1.09	0.84	1.19	3	0.81	1.21
SFA	6	4	0.83	0.65	0.80	0.69	3	0.77	0.75
MUFA	6	1	0.89	0.51	0.87	0.54	4	0.81	0.69
PUFA	7	4	0.83	0.79	0.81	0.85	3	0.80	0.79

LV = latent variable; OUT = outlier; R^2_c = coefficient of determination on calibration set; SEC = standard error on calibration set; R^2_{cv} = cross-validation coefficient of determination on calibration set; SECV = cross-validation standard error on calibration set; R^2_p = prediction coefficient of determination on validation set; SEP = prediction standard error on validation set; FAMES = fatty acid methyl esters; SFAs = saturated fatty acids; MUFAs = monounsaturated fatty acids; PUFAs = polyunsaturated fatty acids.

Acceptable to good predictive performances were recorded for all FAs in cross-validation. The RPD_{cv} (Table 8) was above 3 for nearly all FAs, except for 14:0, 16:0, and the SFA and PUFA classes. In prediction, however, RPD_p dropped below 2.5 for 18:1 cis-11, SFA, and MUFA classes, whereas PUFAs such as 18:2 n-6, 18:3 n-2, and 18:3 n-3 exceeded the threshold of 3, classifying the models as good. This result was influenced by the high variability of the data. Other FAs showed an average RPD_p of 2.66. Additionally, for all FAs, the RER never fell below the threshold of 10.

The slightly lower precision in FA prediction compared to chemical composition is likely due to the more complex and variable nature of FA spectral absorption in the NIR range. Indeed, FA signals are weaker and more influenced by overlapping bands.

Some dairy farms have implemented feeding strategies to improve animal welfare and enrich milk-derived products with nutraceutical compounds. One such strategy involves incorporating a significant percentage of flaxseed, which is rich in n-3 fatty acids, into the diet. This practice has led to an increase in 18:3 n-3 levels, which typically constitute around 3% of the diet, to values exceeding 15% [51]. As n-3 FAs increase, n-6 FAs tend to decrease. NIR prediction models benefit from this high variability in the dataset, allowing the PLSR model to effectively predict variations in certain PUFAs in the TMRs fed to lactating buffaloes.

It has been widely demonstrated in experimental settings that high-yielding dairy animals receiving n-3 FA supplementation in their diet—either in the form of oil or oilseeds—experience metabolic, anti-inflammatory, and reproductive benefits. NIR spec-

troscopy could, therefore, become a valuable tool for monitoring the increased intake of n-3 FAs in the diet [51].

Table 8. Residual Predictive Deviation (RPD) and Range of Reference Values (RER) on cross-validation (cv) and prediction (p) for fatty acid composition (% of total fatty acids) of Total Mixed Rations (TMRs).

%	Calibration Set		Independent Set for Validation	
	RPD _{cv}	RER _{cv}	RPD _p	RER _p
14:0	2.94	15.73	2.97	13.63
16:0	2.84	13.78	2.67	11.59
16:1	3.05	18.42	2.62	18.14
18:0	3.53	21.01	2.52	12.30
18:1 cis-9	3.09	14.85	2.54	13.51
18:1 cis-11	3.16	17.66	2.41	12.36
18:2 n-6	3.01	17.95	3.09	14.80
18:3 n-6	3.88	18.52	3.82	15.83
18:3 n-3	3.02	11.73	3.27	11.37
SFA	2.87	11.41	2.42	10.47
MUFA	3.07	13.81	2.40	11.42
PUFA	2.70	14.36	2.51	12.57

RPD_{cv} = SD/SEC_{cv}; RPD_p = SD/SEP; RER_{cv} = range (max-min value)/SEC_{cv}; RER_p = range (max-min value)/SEP; SFAs = saturated fatty acids; MUFAs = monounsaturated fatty acids; PUFAs = polyunsaturated fatty acids.

When developing NIRS calibration models, it is essential to collect samples that are both relevant and representative of TMR. This requires careful consideration of all potential sources of variability, including formulation-related factors (e.g., concentration ranges of components), physical characteristics (e.g., particle size), and distribution issues (e.g., blending) [8]. A large calibration set that maximises variability in both parameters and ingredients is essential for achieving reliable calibration outcomes. In addition, the method of data acquisition plays a significant role in the accuracy of the results, as noted by several authors [15].

To our knowledge, no studies have specifically developed NIR models for FA content in TMRs. Some research on annual forages [43] has shown excellent calibration results for only a few components, such as 18:3 n-3 ($R^2 = 0.93$, SEC = 1.101, RER = 13.65), total FAs (TFAs) ($R^2 = 0.89$, SEC = 1.854, RER = 11.02), and unsaturated FAs (UFAs) ($R^2 = 0.90$, SEC = 1.431, RER = 11.46). For the other FAs, the models were not satisfactory. Foster et al. [24] reported impressive R^2 values, ranging from 0.93 to 0.99, suggesting that the models developed have a strong fit to the data. Additionally, the reported RPD values greater than 3 indicate good predictive capability, further validating the effectiveness of their approach.

4. Conclusions

This study highlights the potential of NIRS as a rapid and non-destructive analytical tool for assessing the chemical composition and FA profile of TMRs for dairy buffaloes. The benchtop NIRS device demonstrated strong predictive capabilities for key chemical parameters, including dry matter, crude protein, ether extract, and starch, with calibration and cross-validation models achieving R^2 values above 0.90. Predictive models for FA composition showed slightly lower reliability, with R^2 values ranging between 0.80 and 0.90 for most FAs. These models require further refinement, particularly for FAs with lower predictive accuracy.

The study also confirmed that dietary variability, particularly through the inclusion of omega-3-rich ingredients, enhances the robustness of NIR-based FA prediction models. This finding is significant as PUFAs play a crucial role in animal nutrition, not only by

improving the FA composition of animal-derived products but also by exerting metabolic and anti-inflammatory effects on highly productive animals.

Overall, while NIRS presents a promising approach for feed analysis and precision nutrition management in dairy buffalo farming, future advancements in chemometric techniques, dataset expansion, and instrument calibration could enhance its applicability. These improvements would make NIRS an even more reliable tool for evaluating the nutritional quality of TMRs and supporting the sustainable production of buffalo milk.

In future research, it would be valuable to expand the dataset to include a broader range of TMR compositions and feeding strategies across different farming systems. This would enhance the robustness and generalisability of the predictive models, making them applicable to a wider variety of agricultural settings.

Additionally, further work could focus on optimising variable selection to refine the models, particularly when dealing with complex and heterogeneous data. Comparing the performance of different NIRS devices would also provide valuable insights into standardising feed analysis practices.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/app15063211/s1>, Figure S1. Plot of variable contributions in the PLSR models for some compounds; Figure S2. (a–d). Plots of PLSR data analysis for prediction (blue) and cross-validation (red). Each plot corresponds to the best PLSR model obtained for each compound (proximate composition, fibre, fatty acids, and principal classes of fatty acids). Figure S3. Plots of PLSR data analysis for prediction using an independent dataset for all chemical and fatty acid components of TMRs.

Author Contributions: Conceptualisation, S.F. and C.E.; data curation, S.F., C.E. and M.C.; formal analysis, C.E., M.C. and S.F.; funding acquisition, U.B.; investigation, C.E. and S.F.; methodology, S.F., C.E. and M.C.; writing—original draft, C.E. and S.F.; writing—review and editing, L.B. and U.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the European Union NextGenerationEU (grant number J83C22000830005) within the “National Research Centre for Agricultural Technologies” research programme. This manuscript reflects only the authors’ views and opinions; neither the European Union nor the European Commission can be considered responsible for them. PON—Ricerca e Innovazione 2014–2020 (DM1016/2021) is also acknowledged for the Evangelista C. PhD fellowship.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available upon request from the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

MDPI	Multidisciplinary Digital Publishing Institute
DOAJ	Directory of open access journals
TLA	Three-letter acronym
LD	Linear dichroism
TMRs	Total Mixed Rations
GC-FID	gas chromatograph with a flame ionisation detector
NIR	Near-infrared spectroscopy
FT-NIR	NIR with Fourier Transform
SNV	Standard Normal Variate

PLSR	Partial Least Squares Regression
LVs	Latent variables
S.D.	Standard deviation
C.V.	Coefficient of variation
RMSE	Root Mean Square Error
SEC	Standard error on calibration
SECV	Standard error on cross-validation
SEP	Standard error on prediction
R ²	Coefficient of determination for c = calibration, cv = cross-validation, p = prediction
RPD	Residual Predictive Deviation (RPD = SD/SECV or SEP)
RER	Range error ratio ((maximum–minimum)/SECV or SEP)
DM	Dry matter
CP	Crude protein
CF	Crude fibre
EE	Ether extract
aNDF	Neutral Detergent Fibre
ADF	Acid Detergent Fibre
ADL	Acid Detergent Lignin
FA	Fatty acid
FAMES	Fatty acid methyl esters
SFAs	Saturated fatty acids
MUFAs	Monounsaturated fatty acids
PUFAs	Polyunsaturated fatty acids

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