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Suitability of faecal near-infrared reflectance spectroscopy (NIRS) predictions for estimating gross calorific value

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Abstract

A total of 220 faecal pig and poultry samples, collected from different experimental trials were employed with the aim to demonstrate the suitability of Near Infrared Reflectance Spectroscopy (NIRS) technology for estimation of gross calorific value on faeces as output products in energy balances studies. NIR spectra from dried and grounded faeces samples were analyzed using a Foss NIRSystem 6500 instrument, scanning over the wavelength range 400-2500 nm. Validation studies for quantitative analytical models were carried out to estimate the relevance of method performance associated to reference values to obtain an appropriate, accuracy and precision. The results for prediction of gross calorific value (GCV) of NIRS calibrations obtained for individual species showed high correlation coefficients comparing chemical analysis and NIRS predictions, ranged from 0.92 to 0.97 for poultry and pig. For external validation, the ratio between the standard error of cross validation (SECV) and the standard error of prediction (SEP) varied between 0.73 and 0.86 for poultry and pig respectively, indicating a sufficiently precision of calibrations. In addition a global model to estimate GCV in both species was developed and externally validated. It showed correlation coefficients of 0.99 for calibration, 0.98 for cross-validation and 0.97 for external validation. Finally, relative uncertainty was calculated for NIRS developed prediction models with the final value when applying individual NIRS species model of 1.3% and 1.5% for NIRS global prediction. This study suggests that NIRS is a suitable and accurate method for the determination of GCV in faeces, decreasing cost, timeless and for convenient handling of unpleasant samples.

Additional key words: NIR spectroscopy; heating value; faeces

Abbreviations used: GCV (gross calorific value); NIRS (near infrared reflectance spectroscopy); RER (ratio of the range of the original data to standard error of prediction); RPD (ratio of the standard deviation of the original data to standard error of prediction); SD (standard deviation); SEC (standard error of calibration); SECV (standard error of cross-calibration); SEP (standard error of prediction); VC (certified value); VL (laboratory value). **Parameters:** A (accuracy); N_R (total results by sample); r (repeatability); R (reproducibility); r^2 (coefficient of determination of cross validation); R^2 (coefficient of determination of calibration); R^2_{val} (coefficient of determination of external validation); S_r (repeatability standard deviation); S_R (reproducibility standard deviation); U (uncertainty for the half width of the 95% confidence interval; U_R (reproducibility uncertainty).

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Introduction

Energy balances studies serve to assay feed values and to determine animal requirements and efficiencies of feed conversion. They are expensive and time consuming, because to determine the balance between feed input and output products is necessary to quantify the energy received in feed and discarded in faeces, besides in urine (Van Soest, 1994). The measurement of total caloric balance is determined by the gross energy values of feed and faeces using calorimetric bomb through heat exchange under conditions of constant pressure. Faecal samples are a source of information and a cheap material relatively easy to maintain.

Near infrared reflectance spectroscopy (NIRS) has been applied widely and numerous studies have used

NIR spectroscopy to examine the composition and characteristics of faeces (Dixon & Coates, 2009). It is a non-destructive, rapid, economical, flexible and versatile technique. Sample presentation is simple and data (spectra) are collected very rapidly (Sánchez *et al*., 2013). In particular, the benefits associated with NIR analysis of faeces are associated with decreasing cost, timeless and convenience in handling unpleasant samples (Neumeister *et al.*, 1998; Castrillo *et al*., 2005).

Briefly, in NIR spectroscopy it is possible to correlate the light absorbed by the sample with known chemical or physical attribute of the sample. For this reason, the quality of the reference method has a direct effect on the predictive accuracy and precision of the NIR model. A traditional way to ensure the quality of reference data is the knowledge of the uncertainty of measurement results to have an interpretation of the results (ISO/DTS 21748, 2010). The error sources of reference method will be incorporated into the model and could induce erroneous assessment of NIRS performance (Murray, 1993).

To our knowledge, this is the first article dealing with the use of NIRS to predict the gross calorific value of pig and poultry faeces. For this reason, this study sought to demonstrate the suitability of NIRS technology for estimation of gross calorific value on faeces as output products in energy balances studies, with the objective of facilitating the cost-effectiveness and timeliness for routine analysis, establishing the relevance of method performance associated to reference values to obtain an appropriate, accurate and precise NIRS calibration.

Material and methods

Samples and reference data

A total of 220 faecal pig and poultry samples were collected from different experimental trials over an extended period from 2012 and 2013, to attempt the analysis of gross energy. All of them were analyzed in duplicate using a bomb calorimetric IKA®-WERKE C 5000 C (IKA®-Werke GmbH & Co. KG, Germany). The exact determination of the gross calorific value of a substance is based on the requirement that the combustion proceeds under precisely defined conditions. For this purpose, the decomposition vessel is charged with 0.5±0.01 g of representative sample and filled with pure oxygen, after that, it is ignited, and the increase in temperature inside the calorimeter system is measured. The pressure of the oxygen atmosphere in the decomposition vessel is 30 bar. The final gross energy is expressed as cal/g.

To develop NIRS calibration models samples were organised in different sets: Set 1, poultry calibration set including 80 faecal samples; Set 2, pig calibration set including 100 faecal samples; Sets 3 and 4, external validation sets, consisting of 15 and 25 poultry and pig faecal samples, respectively. All the samples included in the validation set were randomly selected.

NIR scanning and calibration procedures

NIR spectra from air-dried faeces samples milled through a 1 mm sieve were analyzed using a Foss NIRSystem 6500 equipped with a monochromator and transport module (Foss NIRSystem, Silver Spring, MD, USA), scanning over the wavelength range 400-2500 nm, every 2 nm. In this study the analysis was carried out using a 5 cm-diameter ring cup. Each sample was measured in two independent subsamples as log (1/R) and after that spectra were averaged. All spectral data were recorded using the WinISI II software v.1.5 (Infrasoft International Inc., Port Matilda, PA, USA).

Population characterization based on spectral variability of the samples was evaluated using CENTER algorithm (Shenk & Westerhaus, 1991a,b; 1995a,b) included in the WinISI II software package, version 1.50 (Infrasoft International, Port Matilda, PA, USA), with a maximum standardised Mahalanobis distance (H) from the average spectrum of 3.0. All NIRS chemometric models were developed using the WinISI II software v.1.5, performing regression analysis with Modified Partial Least Squares, and employing full wavelength range.

The Standard Normal Variate and Detrending methods were applied for scatter correction (Barnes *et al*., 1989), together with first or second derivative as mathematical treatment. Different cross-validation steps were included in the process in order to avoid overfitting of the prediction models. The accuracy of global and single developed calibration models was tested by using cross validation groups. The final calibration model, was also validated externally by applying calibration to unknown samples (Sets 3 and 4), assigning each sample the reference data obtained using the bomb calorimetric.

The final calibration models were selected according to the lowest standard error of cross-validation (SECV), the lowest standard error of prediction (SEP), the highest coefficient of determination of cross validation (r^2), the highest coefficient of determination of external validation (R^2_{val}), and the ratios RPD or ratio of the standard deviation of the original data (SD) to SEP and

RER or ratio of the range of the original data to SEP. These latter two statistics enable SEP to be standardized, facilitating the comparison of the results obtained with sets of different means (Williams, 2001). The best-fitting equations obtained for the calibration set were selected by statistical criteria, and subsequently evaluated by external validation following the protocol outlined by Shenk *et al*. (2001).

Validation statistics

In practice, the approval of the validation studies results demonstrate the reliability of the test method used. Such studies produce data on overall performance and on individual influence factors which can be applied to the estimation of uncertainty associated with the results of the method in normal use. Performance parameters are obtained during method development and interlaboratory study or following in-house validation protocols. Individual sources of error or uncertainty are typically investigated only when significant compared to the overall precision measures in use (CITAC/Eurachem, 2002).

Validation studies for quantitative analytical methods typically determine the following parameters:

— Precision, it measures: (i) repeatability (standard deviation, S_r, indicates the variability observed within a laboratory, over a short time, using a single operator, item of equipment etc.), and (ii) intermediate reproducibility (standard deviation S_R, intermediate precision relates to the variation in results observed when one or more factors, such as time, equipment and operator, are varied within a laboratory) (ISO 5725-1&2, 1994). Repeatability (r) and reproducibility (R) were calculated attending Eqs. [1] and [2]:

$$r = S_r 2\sqrt{2} \quad [1]$$

$$R = S_R 2\sqrt{2} \quad [2]$$

— Accuracy (A), it is usually determined by study of relevant reference materials or by spiking studies. The determination of overall bias with respect to appropriate reference values is important in establishing traceability to recognized standards. A, as percentage, was carried out performing replicate measurements on the reference standard (benzoic acid) under intermediate reproducibility conditions:

$$A = 100 - 100 \cdot \frac{VC - VL}{VC} \quad [3]$$

where VC is the certificate value and VL is the laboratory value.

— Robustness or ruggedness, many protocols for development or validation of methods require that sensitivity to particular parameters be investigated directly. This is usually done by a preliminary 'ruggedness test', in which the effect of one or more parameter changes is observed. Ruggedness test data can therefore provide information on the effect of important parameters.

— Selectivity/specificity, both terms are related to the degree to which a method responds uniquely to the required sample type. The results are normally used to demonstrate that the practical effects are not significant. However, since the studies measure changes in response directly, it is possible to use the data to estimate the uncertainty associated with potential interferences, given knowledge of the results obtained when using different calibrations.

NIRS analysis uncertainty was expressed as a combination of uncertainties,

$$U = \sqrt{SEP^2 + \frac{S_R}{\sqrt{N_R}}^2} \quad [4]$$

where: SEP is the standard error of prediction, which expresses the accuracy of routine results corrected for the mean difference between routine and reference methods (bias); S_R is the standard deviation of the reference results obtained under intra-laboratory reproducibility conditions; N_R is the total results by sample in the test.

Results and discussion

Chemical composition of faeces and method performance characteristics

The mean, range and standard deviation of reference analysis for gross calorific values (GCV) of poultry and pig faecal samples used in this study are given in Table 1. The GCV values for faecal samples of poultry ranged from 3961 to 3312 cal/g, whereas GCV values for faecal pig samples varied between 5174 and 4122 cal/g. As can be seen GCV showed high variability in both animal species, probably because their energy content is the result of the interaction between diet with individual animal digestive physiology (Núñez-Sánchez *et al.,* 2012) and its relationship with the content of undigested residues of the consumed diet (De la Roza *et al.*, 2002).

Table 1. Mean values, standard deviations and ranges for gross calorific values (GCV, in cal/g) of poultry and pig faeces calibration and their validation sets.

Animal species			Maximum	Minimum	Mean	Standard deviation
Poultry	Calibration	Set 1	3961	3312	3671	134.8
Pig		Set 2	5174	4168	4387	188.2
Poultry	Validation	Set 3	3865	3484	3664	123.5
Pig		Set 4	4818	4122	4352	142.1

The accuracy and precision of NIR calibration models are dependent on calibration samples and reference method. Confirmation of performance is desirable from time to time for every analytical instrument. An effective means for detecting erroneous applications and for assuring consistent, reproducible results is to retain a quantity of one or more appropriate materials with known, well-characterized properties which can be used to test instrument performance.

The accuracy and precision of NIR calibration models depends on calibration samples and reference data analysis. The reference method performance is important to have some indication of the quality of the results. Table 2 shows the performance characteristics of the reference method employed to analyse faeces. A percentage of accuracy of 99% was obtained in this study, calibrating the calorimeter with benzoic acid as certified reference material and using a total of 25 measurements carried out in intermediate reproducibility conditions.

The precision was calculated analysing GCV ten times in intermediate repeatability and reproducibility conditions with pig and poultry faeces samples. The overall coefficients of repeatability for these samples were between 46 and 57 cal/g for poultry and pig faeces, respectively, and 66 cal/g for intermediate reproducibility with the same value for both species. These results showed that the reference measurements carried out with bomb calorimetric method were found to be accurate and precise (coefficient of variation of 0.6%).

The uncertainty is the parameter associated with the level of confidence that can be placed in the results. A statement of the uncertainty associated with a result conveys to the customer the quality of the result. The uncertainty value calculated for reference method as a combination of uncertainties for the half width of the 95% confidence interval is 59.25 cal/g, and according with reproducibility results (ISO/DTS 21748, 2010).

Table 2. Performance characteristics of reference method to estimate gross calorific value (in cal/g) on poultry and pig faeces

	Poultry	Pig
S_r	16.1	20.2
S_R	23.2	23.5
Accuracy (%)*	99	99

S_r: repeatability standard deviation; S_R: reproducibility standard deviation. *Benzoic acid has been the reference material for accuracy estimation.

NIR calibration and validation results

After analyzing faeces samples using reference method, different NIR calibration models were assayed. Table 3 reports key statistics on the calibration, cross-validation and external validation to estimate GCV on poultry and pig faeces. The two animal species were considered separately. Both calibrations yielded excellent prediction models for the prediction of GCV after applying second derivative to spectra data. Comparison of the calibration models obtained for both species, found no differences in statistical parameters. The correlation determination coefficients, R^2 and r^2, obtained by chemical analysis and NIRS analysis were, respectively, 0.94 and 0.92 for poultry and 0.97 and 0.94 for pig.

In order to evaluate all the statistical results of the developed models, Williams (2001) recommend that if the sample set is small, the evaluation of the calibration must be done by cross-validation procedure, using parameters such as RER values (Range/SECV). The predictive capacity of the developed models achieved excellent results for all the evaluated parameters, with RER values of 15.94 and 21.03 for poultry and pig respectively.

For external validation, the ratio between the SECV and the SEP varied between 0.73 and 0.86 for poultry and pig respectively, indicating a sufficiently robust calibration (Savenije *et al*., 2006). Assuming the SECV is approximately equal to standard error of laboratory, this ratio is very acceptable with regard to the accuracy of the calibration.

Faeces are an indirect source of information because they are composed of a mixture of undigested material, intestinal flora and endogenous secretions of digestive tract (Van Soest, 1994). Numerous studies have used NIRS to examine the composition and characteristics of faeces, however, few published studies have

Table 3. Statistical results of faecal NIR calibrations and external validation to estimate gross calorific value (cal/g) on poultry and pig faeces

Animal species	Mean	SD	SEC	R^2	SECV	r^2	SEP	R^2_{val}	RPD	RER
Poultry	3677	129.1	31.19	0.94	35.76	0.92	48.63	0.84	2.65	15.94
Pig	4389	187.9	30.26	0.97	46.37	0.94	53.59	0.85	3.51	21.03
Poultry & Pig	4052	356.8	35.92	0.99	47.35	0.98	58.01	0.97	6.15	36.91

SD: standard deviation in reference NIR database; SEC: standard error of calibration; R^2: coefficient of determination of NIR calibration; SECV: standard error of cross validation; r^2: coefficient of determination of cross validation; SEP: standard error of external validation; R^2_{val}: coefficient of determination of external validation; RDP: SD/SEP; RER: Range/SEP

addressed the use of NIR spectroscopy for predicting gross calorific value on faeces. Van den Neucker *et al.* (2002) reported correlation coefficients between results obtained by the NIR and bomb for human faecal samples of 0.91 for monitoring abnormal digestion to identify disease states. In their research study, NIR developed equations offer comparable correlations between NIR predictions and gross energy faecal content and bomb calorimetric. The bias between both methods was −0.134 and −4.960 for poultry and pig faeces, respectively.

Results of different experiments indicate that NIR spectra of faeces can be modified by a variety of factors associated with the species and suggest that NIR calibrations developed using one animal species often cannot be directly applied to predict some attribute from faeces of another animal species (Dixon & Coates, 2009). Our results were broadly in agreement with those reported in the literature. Fig. 1 shows visible+NIR spectra of the pig and poultry faecal populations. These faecal NIR spectra of two species are very similar attending the shape and the alternative of developing a new global calibration needs to be investigated including both populations. However, faeces produced by pig showed higher absorption values than poultry faeces. Despite some spectral regions allowing discriminations among faeces, those bands are associated with the absorption of protein (2050 and 2295 nm); fibre (2275 and 2330 nm) and fat (2350 nm) (Shenk *et al.*, 2001), all of them related with GCV final values (Fig. 1). Probably in their study these differences on spectral regions increased faeces variability to predict energy attributes.

Values for global NIR calibrations and external validation, SEC, SEP and r^2 obtained grouping all faeces samples are shown in Table 3. With this strategy, the new calibration set increases the size and variability of the NIR spectra employed in the development of equation. Because a critical point of NIR spectroscopy for routine analysis is that calibrations should not be used to predict samples from populations outside the limits of range of samples included in calibration data set (Dixon & Coates, 2009). Comparing individual and global NIR statistics, the coefficients of determination, R^2, r^2 and R^2_{val} were 0.99 for calibration, 0.98 for cross-validation and 0.97 for validation, respectively, higher for global than for individual models. In addition also the validation samples caused an increasing in the SD (SD= 363.144), indicating a rise of variability.

The RPD and RER values calculated (RPD 6.15 *vs* 2.65 or 3.51 and RER 36.91 *vs* 15.94 or 21.03) indicated that performance of the equation, with respect to the spread of the data, were better than those obtained with separately equations.

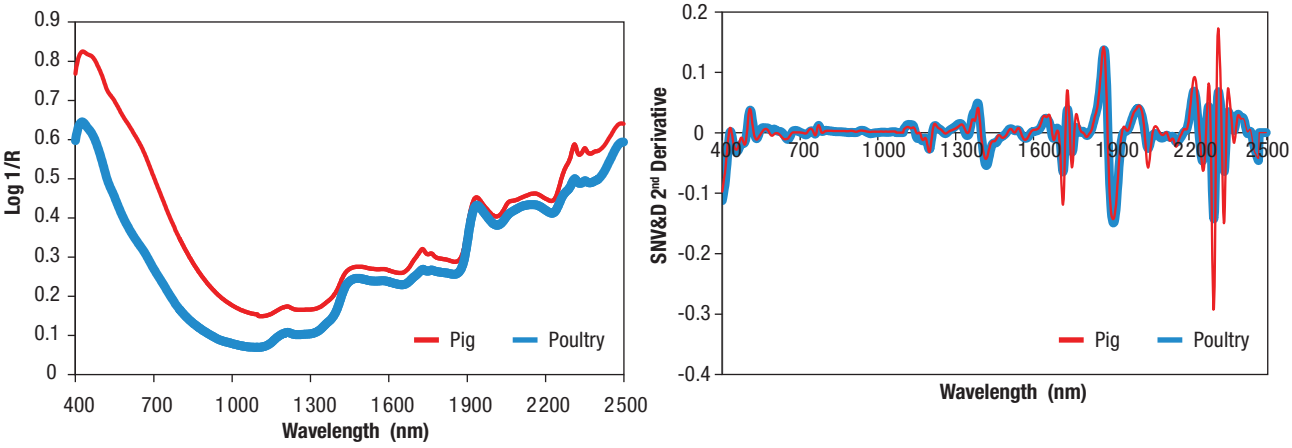

Figure 1. Visible + NIR raw data of the average spectra of pig and poultry faeces samples.

As detailed before, joining both populations as one calibration set the range and variability of final population were increased, and as result coefficient of determination was higher, due to the final value for this statistic is related with the ratio laboratory error/standard deviation of calibration set (Mark & Workman, 1991). However attending to errors (SEC, SECV and SEP) the results show that values are higher using global than individual equations. Summarizing, validation results were: SEP= 58.00, close to global uncertainty of the measurement of reference method; slope= =1.004 and bias= –8.753. This global model could be an additional alternative and it would make possible to use the same model for prediction GCV in both species.

Taking into account all the statistics obtained in the development of the models and the uncertainty expression detailed in material and methods section, in Table 4 includes the uncertainties obtained for each model. As can be seen uncertainty is higher for global model compared with individual NIRS equations. Nevertheless, in terms of relative uncertainty (Urelative) the final value when using individual NIRS species model is 1.3% and 1.5% for NIRS global prediction.

Finally, as reported by Fanchone *et al*. (2009), the main advantage of NIRS compared with other faecal analysis methods is that it takes into account the entire characteristics of faeces through the use of the full NIRS spectrum. With regard to the ease of using NIRS technology and the accuracy and precision of the NIR prediction, as a predictive method for estimating the heat of reaction performed using a bomb calorimeter, an additional benefit could be related with improving the quality of NIRS models, minimizing the error sources of reference method.

As conclusions, these results suggest that residues present in poultry and pig faeces have sufficient spectral information to accurately predict gross calorific values by NIRS as fast technology which allows important savings in time, material and laboratory reagents. It could be demonstrated that if the reference method provide accurate and precise reference data and the spectral data have been subjected to correct chemometric analysis, NIRS calibrations provide low errors.

Table 4. Uncertainties obtained to estimate gross calorific value on poultry and pig faeces for each NIRS assayed model (cal/g)

		Poultry	Pig	Poultry & Pig
SEP		48.63	53.59	58.01
U_R	S_R	23.5	23.2	33.0
	N	10	10	10
U		49.19	54.09	58.94

SEP: standard error of external validation; U_R: reproducibility uncertainty; U: uncertainty for the half width of the 95% confidence interval; S_R: reproducibility standard deviation; N: number of samples.

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