Using Near Infrared Reflectance Spectroscopy (NIRS) to predict the digestible protein and digestible energy values of diets when fed to barramundi, *Lates calcarifer*.

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Abstract

This study examined the potential of using near infrared spectroscopy (NIRS) to predict nutrient digestibility parameters (digestible protein and digestible energy) of compound diets when fed to barramundi. A series of 60 diets were assessed for their protein and energy digestibilities in a series of five experiments over a five-year period from 2009 to 2014. Considerable variance was observed in the digestibility parameters of diets across the experiments, providing a suitable range in diet digestible protein and digestible energy values from which to develop a NIRS calibration. Samples of the same diets were also scanned using a diode array near infrared spectrophotometer (DA-NIRS). The spectra were obtained by the DA-NIRS and were chemometrically calibrated against the digestible value data using multivariate analysis software. The results in terms of standard error of cross validation (SECV), residual prediction deviation (RPD) and correlation coefficient (R^2) show good relationships ($R^2 > 0.8$) between the predicted and observed parameters for both the digestible protein and digestible energy parameters assessed. This study therefore demonstrates that it is possible to use NIRS technology to provide rapid estimates of the digestible protein and digestible energy values of compound diets for barramundi in near real-time.

Introduction

Increasing constraints on the use of fishery resources like fishmeal and fish oils continue to drive pressure on aquaculture feed producers to use alternative raw materials in their formulation of diets for aquaculture species (Tacon & Metian, 2008; Hardy, 2010). In addition to these constraints there is a growing trend to formulating diets for most aquaculture species on a digestible nutrient and energy basis (Glencross et al., 2007). By formulating diets on this basis it allows aquaculture feed producers to adapt to the variability in composition and the nutritional and processing qualities of different raw materials, and consider their nutritional contributions on an equivalent digestible nutrient basis (Glencross et al., 2008; 2011; Velazco-Vargas et al., 2014; Samuelsen et al., 2014). However, obtaining digestibility data is costly, time consuming and limits the ability of feed producers to adapt in near-term time frames to vagaries in the quality of raw materials (Blyth et al., 2014; Diu et al., 2015).

One technology that has gained widespread adoption in feed production for the rapid analysis of the nutritional value of numerous parameters is the use of near infrared spectroscopy (NIRS). Use of this technology to check the chemical specifications of raw materials and complete products (pellets) is now quite routine in many modern aquaculture feed mills due to the perceived reliability and near-real-time turnaround of assessment (Scotter, 1990; Wrigley, 1999; Jiang, 2001; Haughey et al., 2013). Although use of NIRS to assess digestible nutrient and digestible energy parameters is uncommon, it has recently been demonstrated to be possible to determine the digestible protein and digestible energy parameters of single ingredients based on the assessment of their digestibility and development of corresponding calibrations on those derived digestible nutrient and digestible energy specifications of each test ingredient (Glencross et al., 2014). However, despite this development of calibrations for estimating the digestible nutrient and energy parameters of a single raw material type, the development of calibrations for the digestible protein and digestible energy of compound diets, which arguably should be simpler, has not been reported for any aquaculture species.

This study reports on the evaluation of the digestibility of a large number of diets when fed to barramundi (*Lates calcarifer*) and the use of this data set to generate NIRS calibrations for digestible protein and digestible energy. The variability in this data set (digestible nutrient and digestible energy values) was studied using a diode array near infrared spectrometer (DA-NIRS). Based on this DA-NIRS analysis of each diet this study reports on this potential of this technology to predict the digestible protein and digestible energy concentrations of compound diets when fed to barramundi.

Materials and Methods

Experiment concept and diet development

Over a five-year period (2009– 2014), five separate digestibility experiments were undertaken with juvenile barramundi (*Lates calcarifer*). The operational parameters for each trial are detailed in Table 1. The data from these trials, which in many cases focussed on the determination of digestibility data of specific component test raw materials, has been published in other studies (Glencross et al., 2012; 2015; Tabrett et al., 2012; Blyth et al., 2014; Irvin et al., 2015). The present study builds on the data from these experiments and uses that data to test the ability of DA-NIRS to estimate the digestible protein and energy value of compound diets.

Each experiment had a reference diet that was used as the base for each other treatment within each experiment. The formulations and composition of each reference diet is shown in Table 2. The diets from experiments BAR-10-1 and BAR-11-1 were processed by addition of water (about 30% of mash dry weight) to the combined mash during mixing to form a dough, which was subsequently screw pressed using a pasta maker through a 4 mm diameter die. The resultant moist pellets were then oven dried at 70°C for approximately 12 h before being allowed to cool to ambient temperature in the oven. The diets for experiments BAR-09-1, BAR-12-2 and BAR-14-1 were batched and mixed without their oil components before being extruded in a twin-screw extruder according to the conditions described in Glencross et al. (2012). Following extrusion the pellets were oven dried for 12h at 65ºC before being vacuum coated with their respective diet allocations. The effect of diet processing is recognised to impact on digestibility parameters (Glencross et al., 2011b). The use of diets processed by both methods was intentional to exacerbate the range of digestibility values determined for this study.

Fish handling and faecal collection

These digestibility studies constituted five separate *in vivo* experiments. Each experiment was approved (Approval A4/2009) by the CSIRO Animal Ethics Committee according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes 7th Edition, 2004. For each experiment hatchery-reared barramundi (*Lates calcarifer*) were transferred from either BettaBarra (Walkamin, QLD, Australia) or Gladstone Area Water Board Hatchery (Gladstone, QLD, Australia) to experimental holding tanks (10,000 L) where they were on grown for each experiment. Marine water (salinity ~34 PSU) of varying temperatures was supplied to each of 250L or 1000L tanks. For each experiment the tanks were stocked with 20 fish of with initial weights ranging from ~180g to ~440g. Treatments were randomly assigned amongst 24 tanks within each experiment, with each treatment being duplicated, but four replicates achieved through blocking over time.

During each experiment the fish were manually fed the diets each day at 0800 to 0900h, to apparent satiety as determined over three separate feeding events. The fish were allowed to

acclimatise to the allocated dietary treatment for seven days before faecal collection commenced (Blyth et al., 2014). Faeces were collected using manual stripping techniques based on those reported by Blyth et al. (2014). The stripped faeces were collected during 1500 to 1700h over a six-day period, with each fish only being stripped twice and not on consecutive days. Faecal samples collected from different days were pooled within tank, and kept frozen at –20°C before being freeze-dried in preparation for analysis.

Chemical and digestibility analysis

All chemical analyses were carried out according to AOAC (2005) standards. In this regard each of the diet and faecal samples were analysed for dry matter, yttrium, nitrogen and gross energy content. Diets were also analysed for ash and lipid content. The dry matter of each sample was determined by gravimetric analysis following drying in an oven at 105ºC for 24 h. The yttrium concentrations were determined following mixed acid digestion using an inductively coupled plasma mass spectrometry (ICP-MS). Protein levels were determined based on the measurement of the total nitrogen content of each sample using a CHNOS elemental analyser, and a conversion factor of N x 6.25. The total lipid content of the diets was determined gravimetrically following extraction of the lipids using chloroform:methanol (2:1). The gross ash content of each sample was determined gravimetrically following the loss of mass after combustion of a sample in a muffle furnace at 550°C for 12 h. Gross energy content of each sample was determined by ballistic bomb calorimetry. Differences in the ratios of the parameters of dry matter, protein or gross energy relative to the yttrium content, in the feed and faeces in each sample were calculated to determine the apparent digestibility coefficients (ADC_{diet}) for each of the nutritional parameters examined, based on the following formula as reviewed in Glencross et al. (2007):

$$AD_{diet} = \left(1 - \left(\frac{Y_{diet} \times Parameter_{faeces}}{Y_{faeces} \times Parameter_{diet}} \right) \right) \times 100$$

where Y_{diet} and Y_{faeces} represent the yttrium content of the diet and faeces respectively, and $Parameter_{diet}$ and $Parameter_{faeces}$ represent the nutritional parameter of concern (dry matter, protein or energy) content of the diet and faeces respectively.

NIRS scanning and chemometrics

A Diode Array Near Infrared Spectrometer (DA7200, Perten Instruments, Huddinge, Sweden) was used to scan each of the 60 diet samples. These samples were scanned in reflectance mode using the rotating 75mm sample cup. The spectra from all of the samples were collected across the full wave length range (950 to 1650nm) of the instrument as absorbance at a resolution of 2nm using 9 scans per sample (DA7200 Operation Manual, 2007). Each of the scans was collected in groups of 3

with the sample cup being repacked between each group. Each scan was processed by the DA7200 to produce a single spectra profile for analysis. These spectra were then combined with the digestible protein and digestible energy data for each respective diet, which was copied in to the UNSCRAMBLER ® multivariate analysis software package ready for calibration model development. The raw diet spectra as obtained is shown in Figure 1.

All the primary spectra were initially examined visually to eliminate anomalous scans before being copied into the UNSCRAMBLER® multivariate analysis software (Workman and Weyer, 2008). The UNSCRAMBLER® was then used to develop a model that provided a regression based on the whole spectra after SNV pre-treatment and Savitzky-Golay first derivative treatment of the data (Figure 2). The reference digestible protein and digestible energy data was then incorporated to form the calibration data set. Cross validation was then used to evaluate the relationship between the spectra and the digestible protein and digestible energy values. An optimisation program was used to determine the best math pre-treatments and wave number ranges to use with the data that gave the lowest standard error of cross validation (SECV) (Workman & Weyer, 2008). Cross validation tests were subsequently run on the with the whole spectrum pre-treated data. Validation tests were re-run following exclusion of outliers (samples the software flags as either bad reference results or extremely unusual spectrally) (Esbensen, 2004). This process was continued until a balance was determined that included the following elements; a) the SECV that was similar to the standard error of the reference method, b) the number of outliers remaining was small enough, or their residual values are low enough, to still be able to meet the objectives of the calibration, and c) the correlation coefficient (R^2) is sufficiently close to a perfect correlation of 1.0 to indicate probable future robustness and to meet the objectives of the calibration (Esbensen, 2004). Provided the SECV value is in the order of the reference method standard error values of R^2 of 0.6 or even lower can be acceptable but values of over 0.8 are desirable (Workman & Weyer, 2008). For calibration robustness it has been suggested that the standard deviation of the total population used in the calibration model should be at least 1.5x (preferably 2 times or more) the SECV value (Workman & Weyer, 2008). Another method of assessment is the residual prediction deviation (RPD) value, which is the standard deviation of the reference samples divide by the SECV. For RPD values above 10 it is suggested the calibration is about as good as can be expected, whilst values below 2.5 are suggested as being poor (Workman & Weyer, 2008).

Statistical analysis

All values are means unless otherwise specified. Figures were constructed and Multivariate chemometric analysis was undertaken using the UNSCRAMBLER® software (CAMO Software AS, Oslo, Norway).

Results and Discussion

Because variability exists in all raw materials, it is important that strategies are devised to manage this variability, and are used to minimise the impacts of this variability when such raw materials are included in compound diets. If not managed there is a risk of diets not achieving their required specifications and then those diets failing to sustain the growth potential of the animals to which they are fed. The extent of these implications was tested in a study by Glencross et al., (2008) examining the use of lupin meals with varying degrees of digestibility and demonstrated that digestible variability within in a single ingredient can have a significant impact on the performance of fish. Traditionally this management of variability was achieved by the bulk pooling of raw materials to obtain a more homogenous representation of each (Petterson et al., 1999; Jiang, 2001). However, this can be difficult to achieve on a temporal basis and also undermines the potential greater value that can be obtained from higher-quality raw materials through their blending with lower quality ones (e.g. Figure 5). An option to optimise the utilisation of this inherent variability and capitalise on it has been to evaluate the composition and qualities of batches of raw materials and then adapt formulation practices based on this data (Cozzolino et al., 2002). While the use of technologies like NIRS to assist the process have been routine for some time in terms of screening the crude chemical compositional parameters, its use for screening digestible/utilisable parameters of raw materials has been less common (Wrigley, 1999; Kays et al., 2002; Glencross et al., 2014).

In addition to management of the inputs to the formulation process, another option in product quality control is the review of compound diet specification criteria following production. In most modern aquaculture fed mills it is now routine practice to use NIRS to evaluate crude composition specifications of products like; moisture, protein and lipid (Jiang, 2001). However, there are no reports on the use of this technology being used to examine the diet digestible nutrient and energy specifications for aquaculture species. While such an approach doesn't allow the production process to be as reactive as in the case of screening raw material inputs, it does provide a quality control for the output products prior to despatch to the users.

Data variance

Over the present series of five independent experiments a substantial range in the diet composition and digestibility parameters were observed (Table 3). Among the compositional parameters, the most variable was the carbohydrate content which had a coefficient of variation (CV) of 35.8%. This variability was driven by the inclusion of diets in the study which were based largely on only proteinaceous raw materials to those diets which had high inclusion levels of cereal grains and purified non-starch polysaccharides included (Glencross et al., 2012; Irvin et al., 2015). The least variable compositional parameter in the study was that of the dry matter which had a CV of only 2.3%

(Table 3). Protein content of the diets ranged from 396 g kg^{-1} DM to 664 g kg^{-1} DM with a CV of 10.7%, which contrasted that of the energy content of the diets which ranged from only 19.9 MJ kg^{-1} DM to 23.1 MJ kg^{-1} DM with a CV of just 3.6%. This lower variability in the gross energy content reflects the similarity in the energy density achieved with the interchange of protein and carbohydrates. However, this interchange had a more substantive impact on the digestibility of the diets.

The most variable digestibility parameter was that of the dry matter diet digestibilities which had a CV of 19.7% (Table 3). The least variable digestibility parameter was that of lipid digestibility, which had a CV of 5.5%. Diet protein digestibilities ranged from 44.3% to 95.4% and had a CV of 15.0%. Diet energy digestibilities ranged from 45.6% to 85.5% and had a CV of 12.5%. These data are generally consistent with other such data published on diet digestibilities in barramundi (Glencross et al., 2011a; Blyth et al., 2014; Diu et al., 2015; Irvin et al., 2015).

The digestible nutrient and energy parameters are those derived from a combination of the compositional and digestibility ones, therefore they are likely to compound the variability of each (Table 4). The variability in each digestible parameter was compounded by variability in both diet composition and diet digestibilities combining to exacerbate the range of values observed in the present study. Diet digestible protein was the more variable of the two parameters a coefficient of variation of 19.5%, with a range in digestible protein levels of 228 to 587 g kg^{-1} on a dry basis (Figure 3, Table 4). The diet digestible energy levels had a coefficient of variation of 13.4%, with a range in ingredient digestible energy of 9.5 to 18.9 MJ kg^{-1} on a dry basis (Figure 4, Table 4).

NIRS calibration statistics

Although in theory calibrations could be generated for digestibility values, it was deemed more appropriate to focus the present study on the development of calibrations against the digestible characteristics as these represent a more tangible assessment of the nutritional value of the diets (Glencross et al., 2014). Calibrations were successfully developed for both the digestible protein and digestible energy parameters in this study (Figures 3 and 4, Table 4). Among the digestible protein and digestible energy calibrations the number of factors used to derived the calibration varied from 10 (digestible protein) to 7 (digestible energy) (Table 4). The calibration R^2 values ranged from 0.864 for digestible protein to 0.852 for digestible energy. The cross validation R^2 values were closely aligned with the calibration R^2 values, albeit typically a little weaker (Table 4). The standard errors of cross validation (SECV) ranged from 0.8643 for digestible energy to 0.0382 for digestible protein.

The digestible protein and digestible energy calibrations defined within the present paper appear to be quite unique within the scientific literature. Not only are the present calibrations the only such ones found for compound diet digestible value parameters in fish, they also appear to be relatively unique within broader monogastric research. As with other studies recently published in

aquaculture, much of the monogastric NIRS calibration work has focussed on the assessment of discrete component raw materials (van Barneveld et al., 1999; Pujol et al., 2007; Glencross et al., 2014). In contrast to those studies developing calibrations for single raw materials, the present study did not use any cross-experiment reference diet. While it might be argued that the use of a common reference diet may have strengthen the use such datasets across multiple experiments, the present data shows that successful calibrations can still be developed for compound diet digestible nutrient and energy values based solely on those absolute digestibility values. It is doubted whether this could be extended to component raw material evaluations, where a greater degree of cross-experiment fidelity is required because of the increased level of error associated with calculating component raw material digestibilities.

Parameters governing the constraints to an acceptable calibration have been the subject of some debate (Cozzolino et al., 2002; Esbensen, 2004; Workman & Weyer, 2008). However, a common agreement is that they should have a regression R^2 value > than 0.8 and an accuracy >2 times the value reported for the standard deviation of the reference method used to determine that parameter, a value referred to as the RPD (Workman & Weyer, 2008). Clearly both calibrations in the present study had R^2 values exceeding the suggested regression criteria. Using this assessment the digestible protein calibration had a RPD of 2252 and the digestible energy a RPD of 2.4. Therefore this would suggest that the digestible protein calibration is very acceptable, but that the digestible energy calibration still may needs further refinement, despite having a R^2 > 0.80 its RPD value was only marginally below the suggested threshold of 2.5. Importantly, the SECV of the parameters investigated were generally commensurate with the variation in the standard error of each parameter seen across all the diets in this study. Notably the SECV for digestible protein was 0.0382 which was 30 times smaller than the SEM for the same data set. In contrast the SECV for digestible energy was 0.8643, which was three times larger than the SEM for the same data set. As such the RPD values obtained from the present study are at or close to those values considered indicated of robust calibrations for both digestible protein and digestible energy.

Conclusions

The cross validation tests used in this study clearly demonstrate the potential of DA-NIRS to predict the digestible protein or digestible energy values of compound diets when fed to barramundi. Although correlations have been observed between the digestibility values of barramundi and rainbow trout (*Oncorhynchus mykiss*), it would be of value to test the capability of using DA-NIRS to estimate digestible protein and digestible energy for a second species when derived from a calibration such as the present one (Glencross, 2011). An independent study with in vivo and DA-NIRS estimates would enable such a test and should be seen as one priority to follow from the present study.

Acknowledgements

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Tables and Figures

Figure 1. Raw spectral data of compound barramundi diets (n=60) in the NIRS range. Data are not baseline corrected to allow demonstration of data variability more easily. From this figure the overtone regions where most variability was observed can be seen (ca. 1200nm and 1400nm).

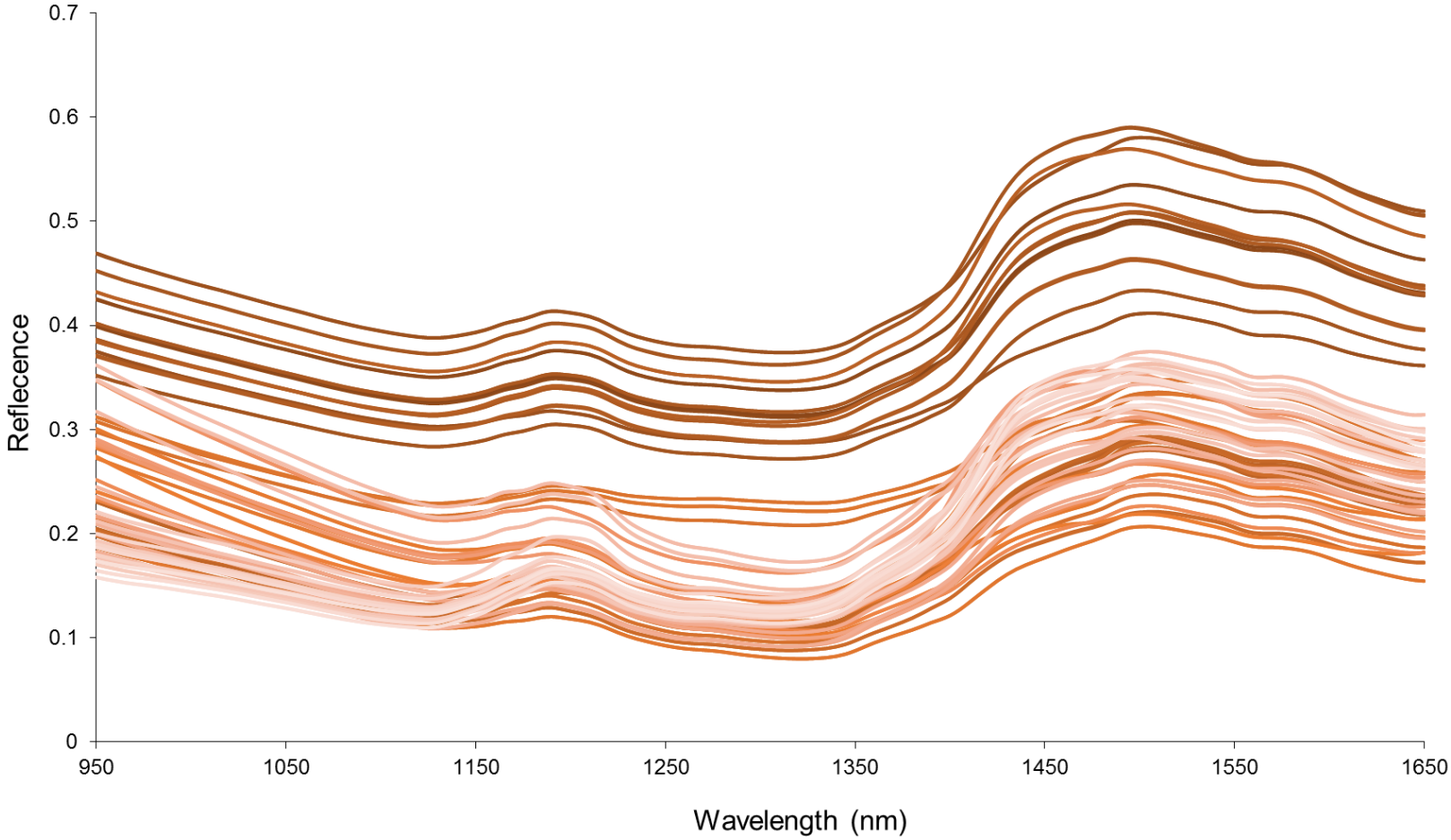

Figure 2. 1st-order derivative of the spectral data of compound barramundi diets (n=60) in the NIRS range. From this figure those overtone regions where most variability was observed can be seen (ca. 1200nm, 1400nm and 1500nm). It is these regions of the spectra that provide most utility in deriving calibrations, but also in many instances relate to specific vibrational modes of certain bond types.

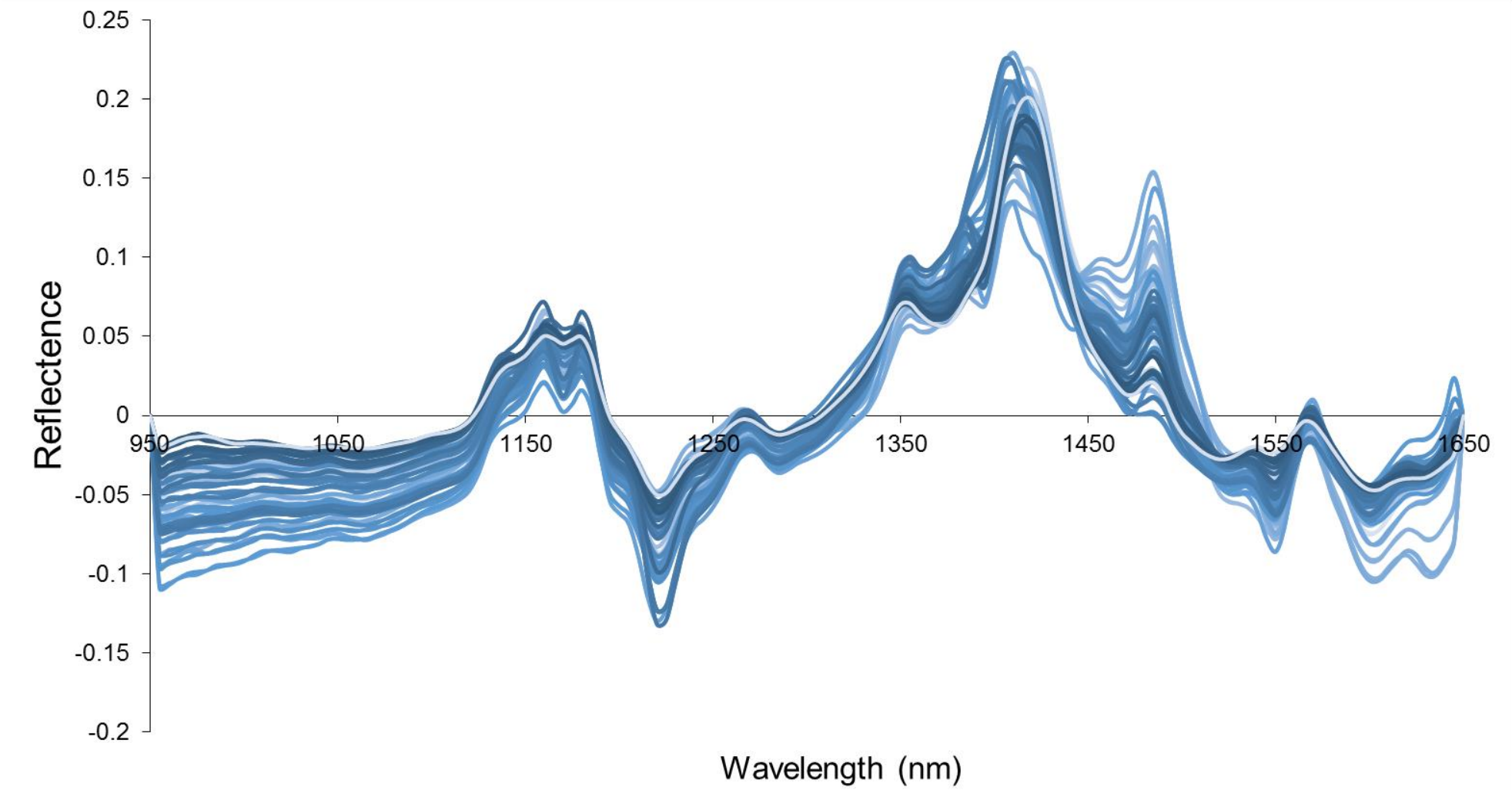

Figure 3. Measured versus NIRS predicted digestible protein value of compound diets in blue data points, with the blue dashed regression line. Shown in red is the cross-validation dataset with the associated red dotted regression line.

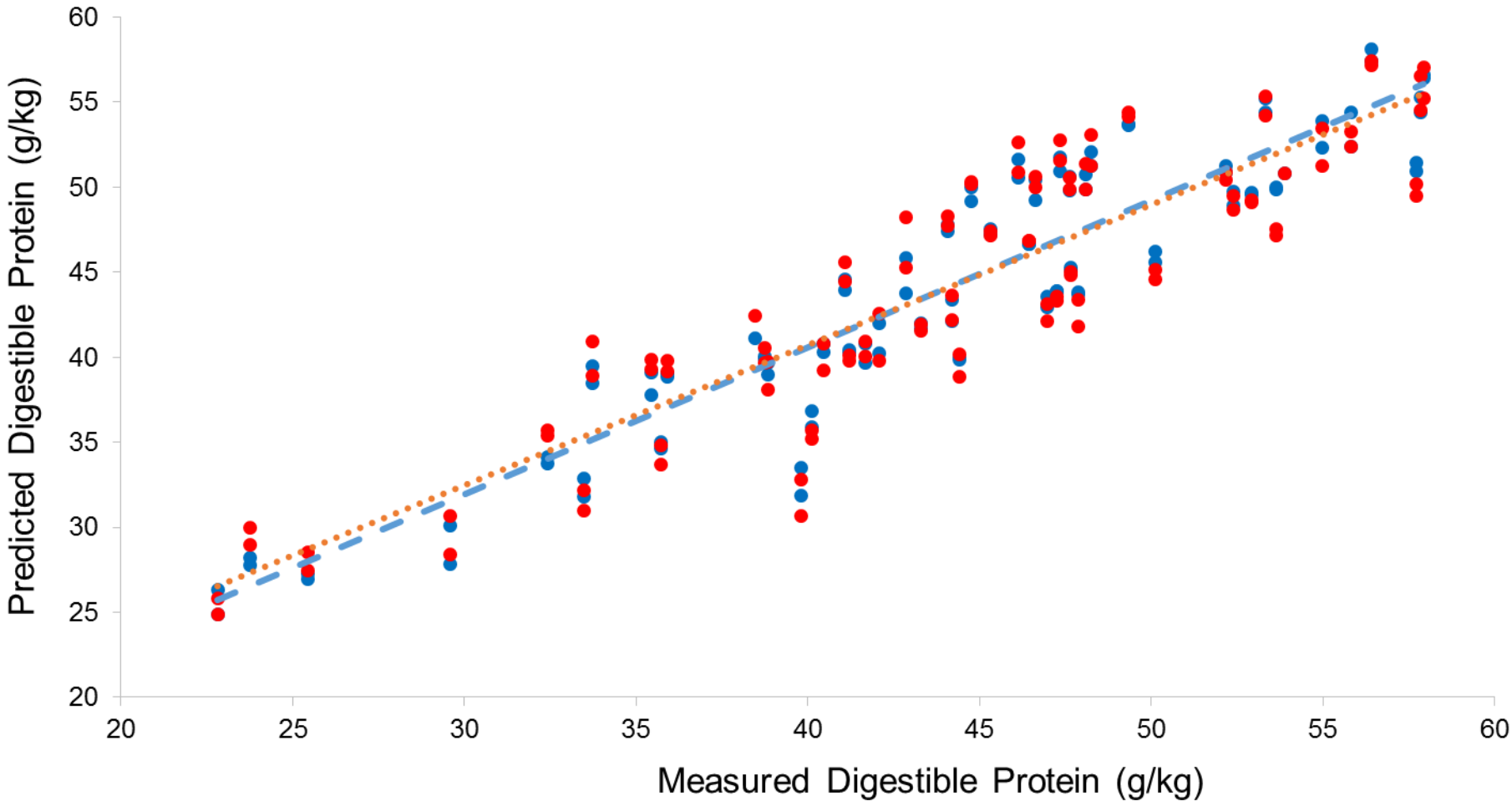

Figure 4. Measured versus NIRS predicted digestible energy value of compound diets in blue data points, with the blue dashed regression line. Shown in red is the cross-validation dataset with the associated red dotted regression line.

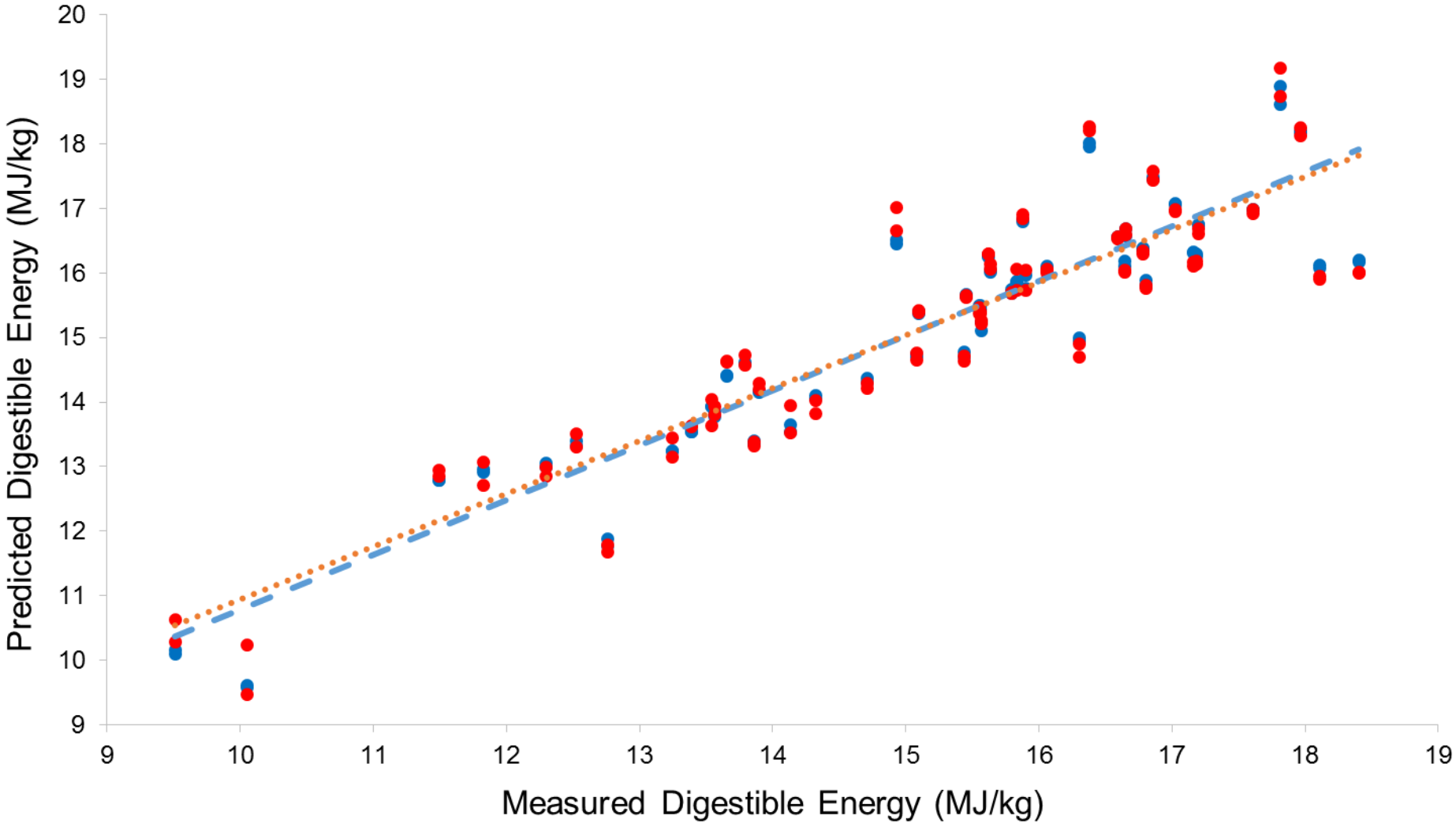

Figure 5. Value of raw materials (f.o.b. port of origin) against their crude protein content. Data is based on January 2014 prices.

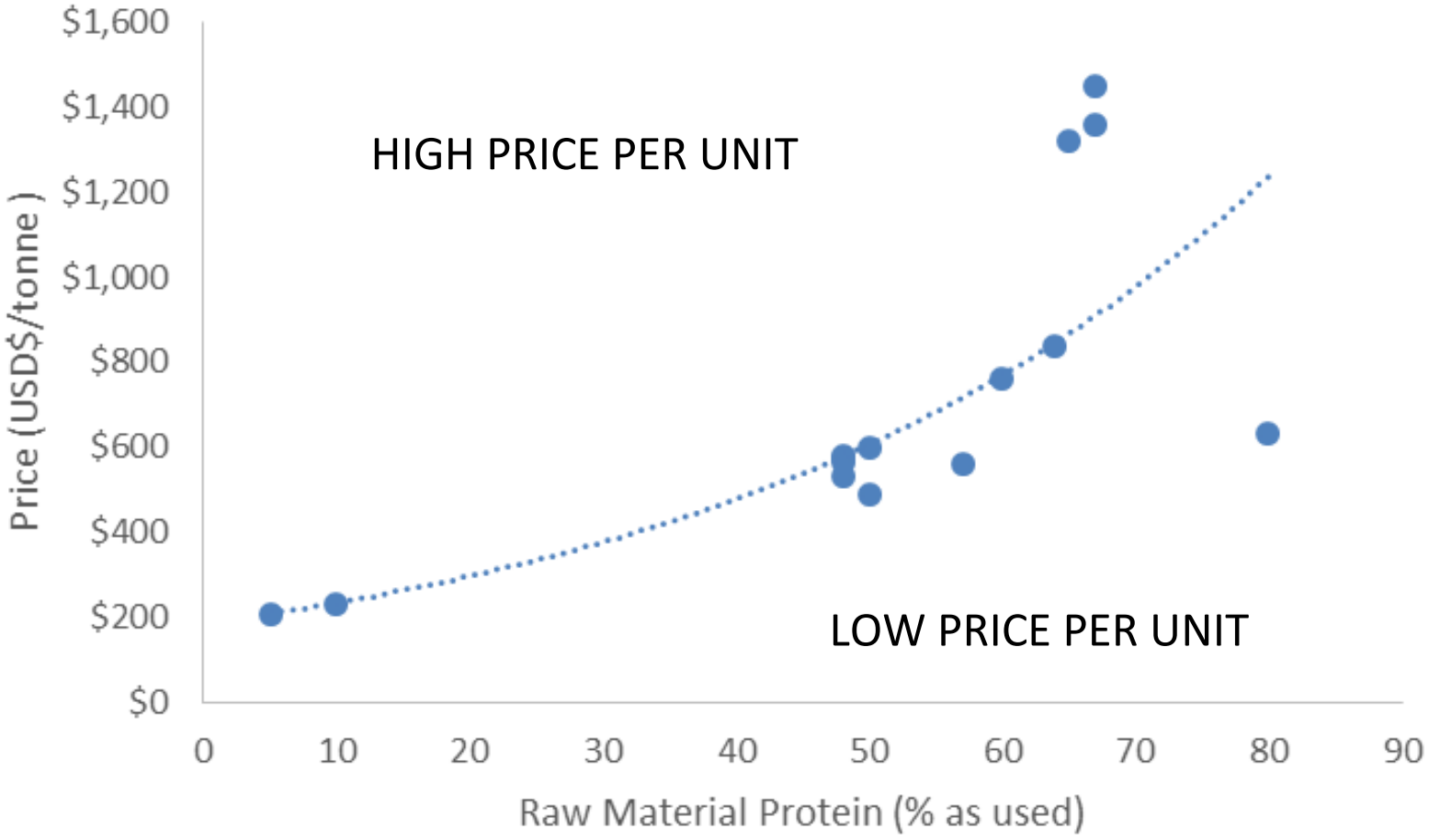

Table 1. Digestibility experiment operational parameters data

Experiment	Temperature ºC	DO mg L^{-1}	Tank Volume L	Fish Weight g $fish^{-1}$	Published as
BAR-09-1	~30	~6.0	250	192 ± 39.0	Glencross et al., 2012
BAR-10-1	28.8 ± 0.22	6.4 ± 0.15	250	398 ± 68.8	Blyth et al., 2014
BAR-11-1	28.6 ± 0.20	6.2 ± 0.2	250	398 ± 68.9	Irvin et al., 2015
BAR-12-2	29.9 ± 0.12	5.5 ± 0.56	250	179 ± 73.0	Glencross et al., submitted
BAR-14-1	30.3 ± 1.50	6.2 ± 0.1	1000	439 ± 97.2	Glencross et al., submitted

Table 2. Reference diet formulations for each experiment

Experiment	BAR-09-1	BAR-10-1	BAR-11-1	BAR-12-2	BAR-14-1
Fishmeal (anchovetta)	640	764	640	764	750
Fish oil (anchovetta)	100	50	100	50	20
Wheat flour	-	80	130	80	224
Cellulose	124	100	-	100	0
*Vitamin and mineral premix	5	5	5	5	5
Wheat gluten	130	-	-	-	-
Pregelled wheat starch	-	-	124	-	-
Yttrium oxide	1	1	1	1	1

* Vitamin and mineral premix includes (IU/kg or g/kg of premix): Vitamin A, 2.5MIU; Vitamin D3, 0.25 MIU; Vitamin E, 16.7 g; Vitamin K,3, 1.7 g; Vitamin B1, 2.5 g; Vitamin B2, 4.2 g; Vitamin B3, 25 g; Vitamin B5, 8.3; Vitamin B6, 2.0 g; Vitamin B9, 0.8; Vitamin B12, 0.005 g; Biotin, 0.17 g; Vitamin C, 75 g; Choline, 166.7 g; Inositol, 58.3 g; Ethoxyquin, 20.8 g; Copper, 2.5 g; Ferrous iron, 10.0 g; Magnesium, 16.6 g; Manganese, 15.0 g; Zinc, 25.0 g.

Table 3. Diet composition and digestibility parameters across all experiments and diets (n=60)

	mean	SD	CV%	maximum	minimum
Composition parameters					
Dry matter (g kg^{-1} as fed)	951	22	2.3	985	882
Protein (g kg^{-1} DM)	543	58	10.7	664	396
Lipid (g kg^{-1} DM)	124	38	30.7	215	61
Carbohydrate (g kg^{-1} DM)	226	81	35.8	444	11
Ash (g kg^{-1} DM)	107	25	23.8	174	72
Energy (MJ kg^{-1} DM)	21.3	0.8	3.6	23.1	19.9
Digestibility parameters					
Dry matter (%)	57.3	11.3	19.7	74.4	27.2
Protein (%)	81.9	12.3	15.0	95.4	44.3
Lipid (%)	89.9	5.0	5.5	96.6	73.3
Starch (%)	70.8	12.6	17.8	93.9	49.1
Energy (%)	72.0	9.0	12.5	85.5	45.6

Table 4. NIRS calibration statistics

Sample characteristics								*Calibration statistics*				
Parameters	n	Mean	SEM	SD	CV%	Max	Min	Factors	Cal R^2	Val R^2	SECV	RPD
Digestible Protein	60	442	11.6	86	19.5	587	228	10	0.864	0.806	0.038	2252
Digestible Energy	60	15.2	0.28	2.0	13.4	18.9	9.5	7	0.852	0.821	0.864	2.4