Journal homepage: http://www.sasas.co.za/journals

South African Journal of Animal Science 2018, 48 (No. 5)

Prediction of *in vivo* organic matter digestibility of ruminant feeds using *in vitro* techniques

K-J. Leeuw[1#], D. Palić[2], F.K. Siebrits[3], H. Muller[4] & V.A. Hindle[5]

[1] Agricultural Research Council, Private Bag X2, Irene, 0062, South Africa
[2] Institute for Food Technology, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia
[3] TUT, Department of Animal Sciences, P. Bag X680, Pretoria 0001, South Africa
[4] UNISA Department of Statistics, P O Box 392, Unisa 0003, South Africa
[5] Wageningen UR Livestock Research, PO Box 65, 8200AB Lelystad, Netherlands

(Received 9 July 2014; Accepted 23 July 2018; First published online 20 November 2018)

Abstract

Prediction equations derived from *in situ* and *in vitro* analytical techniques to determine *in vivo* organic matter digestibility (OMD) are useful tools to estimate the quality of livestock feed. Most derived equations are aimed at groups of feedstuffs (forages or concentrates) or feeds separately. In this study of OMD, the prediction equations of the modified two-stage Tilley & Terry *in vitro* technique (MT) and pepsin-acid multi-enzymatic technique (PME) are compared, validated, and improved in relation to verified *in vivo* results using feedstuffs and complete diets. Initial comparison with *in vivo* data showed that the combined dataset and that of single feedstuffs achieved acceptable R^2 values for both MT and PME (0.88 and 0.92, 0.87 and 0.89, respectively). The validation with the second dataset established that the initial equations were valid with R^2 values of 0.96 for MT and 0.91 for PME on the combined feeds dataset. The establishment of a prediction equation using both datasets resulted in improved R^2 values over the initial equation. With combined feeds using MT it was 0.94, compared with 0.88, and using PME, it was 0.91 compared with 0.87. No significant decrease occurred in the variation of OMD between the datasets, as explained by the model when omitting on separate slope and intercept, thus confirming the same population assumption. The data sets could be combined for a new prediction equation. The R^2 values were 0.94 and 0.91 for MT and PME methods for combined feeds, respectively. The new improved *in vivo* prediction equation in each instance was thus valid and a true improvement on the initial prediction equations. The PME method can be used for predicting OMD as it negates the use of rumen liquor and confidently replaces MT OMD determinations.

Keywords: Modified two-stage *in vitro*, multi-enzymatic, pepsin acid, rumen liquor, validation
Corresponding author: kleeuw@arc.agric.za

Introduction

Feed cost is a major variable in raising or finishing livestock in a farming enterprise. Great emphasis is therefore placed on the quality of animal feed to improve feed efficiency and therefore reduce feed cost per production unit. An important determinant of feed quality is the digestibility of nutrients. The most accurate way of obtaining information about the digestibility of organic matter of feed for ruminants is by conducting *in vivo* digestibility studies. Since these methods are expensive and time consuming, and are not suited to routine analysis, reliable laboratory methods should be developed for routine prediction of the *in vivo* organic matter digestibility (OMD) of ruminant feeds (Beecher *et al.*, 2015).

Although the *in situ* and *in vitro* techniques have good potential to predict *in vivo* OMD (Khazaal *et al.*, 1993; Chenost *et al.*, 2001), they have not been fully validated (Gosselink *et al.*, 2004). Additionally, most developed techniques have been used to estimate the OMD of forages (Beecher *et al.*, 2015; Gierus *et al.*, 2016). There were only a few reports on the use of *in vitro* methods to estimate the OMD of compound feeds and complete diets (Aufrère & Michalet-Doreau, 1988). In addition, the results of OMD prediction for feedstuffs and for compound diets for ruminants are seldom reported in a single study (Dowman & Collins, 1982).

URL: http://www.sasas.co.za
ISSN 0375-1589 (print), ISSN 2221-4062 (online)
Publisher: South African Society for Animal Science
http://dx.doi.org/10.4314/sajas.v48i5.10

There are limitations to the use of rumen liquor for digestibility studies. There must be fistulated animals, which are not available to all laboratories, to collect fresh rumen liquor. Rumen liquor cannot be cooled down and must stay anaerobic (Stern *et al*., 1997). These animals must be maintained on a standard feeding regime to minimize changes in the rumen microbe population (Jones & Theodorou, 2000).

Feeds can be incubated with enzymes to predict *in vivo* OMD. This process aims to mimic the digestive process in the animal. The use of enzymes makes the analyses completely independent of the animal (Stern *et al*., 1997). Most enzymatic methods for OMD estimation were developed for forage feedstuffs, with a few being used for compound feeds (Aufrère & Michalet-Doreau, 1988; Weisbjerg & Hvelplund, 1993). Aufrère & Michalet-Doreau (1988) used two enzymatic methods (digestion by pepsin-cellulase, with 1 N HCl or 0.1 N HCl) to estimate the OMD of compound feeds. These were adapted from the enzymatic method, which were developed for single forages. They found that the estimation of digestibility was better with 0.1 N HCl than that of other chemical or biological methods. Weisbjerg & Hvelplund (1993) developed a pepsin-acid multi-enzymatic incubation method to estimate the enzymatic digestibility of organic matter for use on compound feeds. This procedure also showed the ability to estimate the OMD of straws (Hvelplund *et al*., 1999), and thus demonstrated the potential of this method to predict the *in vivo* OMD of both complete diets and forages.

The aim of this study was to verify the validity of the pepsin-acid multi-enzymatic procedure of Weisbjerg & Hvelplund (1993) to accurately predict *in vivo* OMD with a wide range of feedstuffs and compound feeds for ruminants and to compare these results with existing modified two-stage *in vitro* technique of Tilley & Terry (1963) and *in vivo* procedures.

Material and Methods

The *in vivo* OMD of all feedstuff and compound feed samples was determined in trials with sheep. The trials were conducted at the Animal Sciences Group of Wageningen UR, Division: Animal Production (former Institute for Animal Science and Health, ID-DLO, Lelystad), The Netherlands. The Animal Ethics Committee at the Animal Sciences Group of Wageningen UR, Division, approved the trial protocol: Animal Production and TUT (ref. number: AREC2011/06/008). Rumen liquor for modified Tilley & Terry *in vitro* analysis was collected from cannulated sheep housed at ARC-API Irene, approved by ARC–API Animal Ethics Committee.

To develop the initial prediction equations, 17 samples of commonly used temperate feedstuffs (including maize bran, maize gluten, maize gluten silage, maize cob leaves, hominy chop, barley, wheat, wheat middlings, wheat bran, wheat straw, sunflower oilcake, soybean meal and cottonseed meal) and six complete diets (with pre-determined *in vivo* OMD were used. An additional 21 samples of temperate feedstuffs and 24 complete diets were used to verify the prediction equations. Therefore, 68 samples of ruminant feeds were used to compare the *in vivo* OMD of ruminant feedstuffs, complete diets and their combination with *in vitro* analysis and the PME method.

All samples were analysed for OMD using the PME OMD procedure (Weisbjerg & Hvelplund, 1993) and a modified *in vitro* two-stage OMD technique of Tilley & Terry (1963) (MT). Multi-enzymatic OMD values were compared with the apparent OMD values determined *in vivo*, and with values established with MT as this is currently the preferred method for estimating OMD of ruminant feeds.

The modified two-stage *in vitro* OMD (Tilley & Terry, 1963) was followed. The sample (0.5 g, ground in a Wiley mill through 1 mm sieve) was placed in 100 mL Schott reagent bottles (warmed to 39 °C). To this were added 5 mL urea solution (8.6 g to 1 L distilled water, as adapted by Engels & Van der Merwe (1967), and 50 mL rumen-saliva mixture (1 L rumen fluid mixed with 2 L McDougall's artificial saliva modification done by the ARC–API nutrition laboratory to improve repeatability), while flushing bottles with CO_2 gas to maintain an anaerobic environment. The bottles were then sealed and placed in an incubator (39 °C) and swirled at regular intervals (24 hours). After 48 hours incubation the sample was removed from the incubator, the lid was removed and 5 mL HCl solution (600 mL HCl to 400 mL distilled water or 6N HCl) was added in small volumes (1 mL then swirl, 2 mL then swirl, and 2 mL and swirl), after which 5 mL pepsin solution (8 g pepsin (2500 units/g) to 1 L distilled water) was added. The bottles were then sealed and placed in an incubator at 39 °C, and swirled at regular intervals (24 hours). After another 48-hour incubation, the samples were removed from the incubator. The contents of the Schott bottles were carefully transferred to a glass centrifuge tube (100 mL) after centrifuging (1207.4 relative centrifugal force (RCF) for 10 min.) the supernatant was removed. The residue was rinsed with distilled water and centrifuged again (1207.4 RCF for 10 min.). The tube and contents were dried for 24 hours at 105 °C after which the tube and sample were weighed. After this the tube and sample were placed in a furnace for 6 hours at 500 °C, and then both were placed in a desiccator to allow for cooling down (30 min.) prior to being weighed.

Dry matter and ash contents of feeds were determined and used to calculate *in vitro* digestibility of organic matter for both MT and PME OMD analysis. Organic matter digestibility of the samples determined *in vitro*, were calculated as:

In vitro organic matter digestibility (%) =

$$= \frac{\text{g organic matter in sample} - \text{g insoluble organic matter}}{\text{g organic matter in sample}} \text{ x } 100$$

Castrated adult male sheep (Texel and Texel crosses) with average bodyweight of about 75 kg were used. Animals were housed in special-purpose balance crates under controlled room conditions in the purpose-built metabolism unit. The balance crates are designed to facilitate separation of faeces and urine and allow their separate collection. Water was freely available at all times. The daily amount fed to each animal (two equal portions at two mealtimes) was standardized at 1000 g DM daily for each animal. Animals went through an adaptation period of two weeks, then an 11-day preliminary period, during which the animals were fed the trial ration (feed residues and faeces were not collected), after which the 10-day collection period followed, during which the exact amounts of feed, feed residues and the faecal production were recorded. Feed residues and faeces were collected quantitatively for each animal. At the end of the trial, total feed residues and faeces were homogenized and weighed. Subsamples of feed, feed residues and faeces were taken to determine dry matter and organic matter according to the AOAC (2002) methods. During the preliminary period of each trial, a composite sample of feeds was taken for OMD analysis. Collected samples were sent by courier to South Africa for the MT and PME analysis.

In vivo OMD was calculated using the following equation:

In vivo organic matter digestibility (%) = (feed dry matter consumed x % organic matter feed) – (faeces dry matter produced x % organic matter faeces) = organic matter disappeared / (feed consumed x % organic matter feed)

The *in vivo*, PME OMD, and the MT OMD values were analysed, using SAS (Statistical Analysis System) software package (SAS Institute Inc., 1989, V 8), to evaluate how well the two methods predicted *in vivo* measures. Regression functions and R^2s (with associated mean square error values) for an initial population of 6 complete diets and 17 feedstuffs and for a second population of 24 complete diets and 21 feedstuffs were derived for each OMD procedure, using SAS. The OMD values of the 68 feeds, obtained by both PME OMD and MT procedures, were linearly regressed against the *in vivo* OMD values. Verification and improvement of the regressions were done using the first dataset versus the second dataset. The formula used for the verification regression was:

$$y_i = \beta_0 + \beta_1 x1_i + e_i$$

where: y_i represents the estimated *in vivo* values for OMD
x_{1i} represents the calculated MT or PME method OMD of the second experiment (and ß$_0$ and ß$_1$ are set to the intercept and slope estimates)
e_i represents the random error component.

The same population test on both datasets was done using a multivariate regression approach in SAS. Further differences for slope to unity were also tested under H0: $\hat{\beta}_1 = 1$ after lack of fit was determined.

Results and Discussion

The first dataset comprised six complete sheep diets and 17 feedstuffs (data not shown). The general equation for predicting OMD can be expressed as:

$$y_i = \beta_0 + \beta_1 x1_i + e_i$$

where: y_i represents *in vivo* organic matter digestibility
x_{1i} represents the modified *in vitro* or multi-enzymatic method
e_i represents the random error component and the slope by β_1 and intercept by β_0

The results of the regression equations based on the digestibility values (not shown) for the first dataset are presented in Table 1.

Table 1 Organic matter digestibility means and regression equations for complete diets selected feedstuffs and combined for modified *in vitro* and pepsin-acid multi-enzymatic technique for the first dataset

Feed	Method ($x1_i$)	OMD Mean[1]	Parameter estimates; f-probabilities, significance				
			df error (N)	Regression F-prob.	Intercept β_0	slope β_1	R^2
	In vivo	685					
6	MT	684	4 (6)	0.03*	-17.4 ns	0.98*	0.75
	PME	684	4 (6)	0.01**	102.0 ns	0.82**	0.86
	In vivo	760					
17	MT	770	14 (16)	<0.0001***	154.2**	0.80***	0.92
	PME	760	14 (16)	<0.0001***	249.4***	0.67***	0.89
	In vivo	740					
23	MT	747	20 (22)	<0.0001***	112.3*	0.84***	0.88
	PME	740	21 (23)	<0.0001***	218.5***	0.70***	0.87

[1] Dependent means for modified Tilley & Terry (MT) and pepsin-acid multi-enzymatic (PME) methods, organic matter digestibility (OMD), *in vivo* mean is true mean; 'Regression F-prob' refers to the probability associated with the overall regression F-statistic; Significance legend: *5%; **1%; ***0.1% level of significance; ns: not significant

The prediction equations, based on both analytical methods, predict *in vivo* measurement or value reliably (the R^2 values vary between 0.75 and 0.92 on the 0.1% level of significance, Table 1). These initial equations were followed by a second experiment in which a further 21 feedstuff samples and 24 complete diet samples (45 sample sets in total) were measured OMD *in vivo*, MT and PME. This was done to verify and improve the prediction equations and the predictive power of the first experiment. It was argued that if this first set of predictions equations (Table 1) were reliable, accurate estimates of the measured *in vivo* values (determined for the second experiment) should be obtained if calculated values for the MT PME (calculated in the second experiment) were substituted in the prediction equations derived in the first experiment. Therefore, these estimated *in vivo* OMD, using prediction equations in Table 1 for feedstuff, combined feeds and complete diets, were then regressed against the observed *in vivo* values for the second dataset, which resulted in the regression equations reported in Table 2. The statistically significant R^2 coefficients reported in Table 2 (values range between 0.50 and 0.97 on the 0.1% level of significance) describe the close relationship between the new observed *in vivo* values and the estimated *in vivo* values based on the prediction equations of the first experiment. This thus served as a measure of the predictive power of the initial prediction equations. The exception was the complete diets with R^2 values of 0.50 and 0.75, which was understandable, given that the initial prediction for complete diets were based on six values. The second experiment aimed to improve the prediction equation for complete diets by including 24 new samples.

The formula used for the verification regression:

$$y_i = \beta_0 + \beta_1 x1_i + e_i$$

where: y_i represents the estimated *in vivo* values for OMD
x_{1i} represents the calculated modified *in vitro* or PME method OMD of the second experiment (and $ß_0$ and $ß_1$ are set to the intercept and slope estimates listed in Table 1
e_i represents the random error component.

The R^2 values (Table 2) indicate that the previous prediction equation (Table 1) predicted the new data accurately for the feedstuffs and the combined dataset. The new dataset for complete diets (24 samples) did not fit the first dataset (6 samples), as was evident from the low R^2 values (Table 2) at 0.49 and 0.73 for the MT and PME techniques, respectively. The low number of samples may have contributed to the low R^2 value.

Table 2 Predicted *in vivo* organic matter digestibility mean values, predicted with estimates of modified *in vitro* or pepsin-acid multi-enzymatic values of first dataset, regressed against observed *in vivo* values second dataset: complete diets, selected feedstuffs and combined feeds

Feed	Method ($x1_i$)	OMD Mean[1]	Parameter estimates; f-probabilities, significance				
			df error (N)	Regression F-prob.	Intercept β_0	slope β_1	R^2
	In vivo	824					
24	MT	842	22 (24)	<0.001***	378.4***	0.557***	0.49
	PME	842	22 (24)	<0.001***	369.4***	0.541***	0.73
	In vivo	662					
21	MT	668	17 (19)	<0.0001***	-189.2***	1.26***	0.97
	PME	662	19 (21)	<0.0001***	-220.5***	1.25***	0.94
	In vivo	755					
45	MT	755	41 (43)	<0.0001***	-87.9**	1.13***	0.96
	PME	748	43 (45)	<0.0001***	118.2***	1.12***	0.94

[1] Dependent means for modified Tilley & Terry (MT) and pepsin-acid multi-enzymatic (PME) methods, organic matter digestibility (OMD), *in vivo* mean is true mean; 'Regression F-prob' refers to the probability associated with the overall regression F-statistic: Significance legend: *5%; **1%; ***0.1% level of significance

Table 3 Improved organic matter digestibility means and regression equations to estimate *in vivo* measurement in complete diets, selected feedstuffs and combined, for modified *in vitro* and multi-enzymatic technique method

Feed	Method ($x1_i$)	OMD Mean[1]	Parameter estimates; f-probabilities, significance				
			df error (N)	Regression F-prob.	Intercept β_0	slope β_1	R^2
	In vivo	796					
30	MT	796	28 (30)	<0.001***	8.80^{ns}	0.97***	0.82
	PME	796	28 (30)	<0.001***	224.3^{***}	0.66***	0.90
	In vivo	707					
38	MT	715	33 (35)	<0.0001***	49.9*	0.94***	0.95
	PME	706	36 (38)	<0.0001***	132.3***	0.83***	0.92
	In vivo	746					
68	MT	752	63 (65)	<0.0001***	56.1**	0.92***	0.94
	PME	746	66 (68)	<0.0001***	160.6***	0.75***	0.91

[1] Dependent means for modified Tilley & Terry (MT) and pepsin-acid multi-enzymatic (PME) methods, organic matter digestibility (OMD), *in vivo* mean is true mean; 'Regression F-prob' refers to the probability associated with the overall regression F-statistic; Significance legend: *5%; **1%; ***0.1% level of significance; ns: not significant

The argument was made that the initial *in vivo* prediction models, obtained with the first dataset and verified with the second, could be refined and improved on by calculating new prediction models for *in vivo* estimations (using either the MT or PME) based on the combined dataset of the first and second experiment of this study. The results of improved *in vivo* prediction models for feedstuffs, complete diets and combined feeds are reported in Table 3. Models are reported for both MT and PME.

Table 3 reports R^2 values that range between 0.82 and 0.95 on the 0.1% level of significance, which bears evidence to the strong predictive power of both the MT and PME techniques to estimate *in vivo* measurements. Although the combined dataset gives an improved R^2 (Table 3) value, the improved equation could be validated in future research with new datasets.

The regression equations in Table 3 indicate that compared with the R^2 values of Table 1 and Table 2 there is an improvement in the accuracy of the predictive equations.

The argument further had to be addressed whether it was justified to combine the two datasets: in other words, whether data from the two datasets came from the same population. This issue was addressed by means of a multivariate regression approach (including a dummy variable): a linear *in vivo* regression was calculated for combined feeds (with either MT or PME values as independent variable) for the combined data of datasets one and two. Furthermore, a dummy variable was then entered into the equation to test for the effect of separate, statistically significant slopes for datasets one and two. A second dummy variable was lastly entered into the model to test for the effect of separate, statistically significant intercepts for the datasets. It was reasoned that if the effect of slope proved statistically significant, this would suggest that the two datasets came from different populations and that the improvement of the prediction equations, by combining the datasets were not justified.

To accommodate the evaluation of separate slopes and intercepts in the regression model, a 'zero/one' variable was introduced into the dataset: for the combined data, values of 0 identified the first dataset and a value of 1 the second dataset. The prediction equation when testing the same population assumption for the OMD of combined feeds (y_i) for the MT or the PME methods ($x1_i$), can be expressed as:

$$y_i = \beta_0 + \beta_1 x1_i + \beta_2 x2_i + \beta_3 x1_i x2_i + e_i ; I = 1\ldots\ldots n$$

where: $x1_i$ represents the MT or PME method observations
$x2_i$ represents the qualitative variable with values of 0 and 1 to identify an observational value as belonging to the first or second dataset
β_0, β_2 represents intercept-component parameter estimates
β_1, β_3 represents slope-component parameter estimates
e_i represents the random error component

The regression model can be broken down into a prediction model for the first and second datasets by plugging in the values of $x2_i$. For the first dataset observations, with $x2_i = 0$, the prediction equation for OMD becomes:

$y_i = \beta_0 + \beta_1 x1_i + e_i$; i = 1……n; with a slope of β_1 and an intercept of β_0.

when $x2_i = 1$, representing the new dataset values, the prediction equation becomes:

$y_i = (\beta_0 + \beta_2) + (\beta_1 + \beta_3)x1_i + e_i$; i = 1……n; with a slope parameter of $(\beta_1 + \beta_3)$, and an intercept parameter of $(\beta_0 + \beta_2)$ (Table 4).

The determination of the validity of the same population assumption of the improved *in vivo* prediction equations was concluded with an investigation of the residuals of both prediction equations to ensure the normality of residuals and group-homogeneity of residual variances (assumptions of regression). The results are reported in the last row of Table 4. The tests indicated that these assumptions were complied with.

From Table 4 it can be deduced that the effect of separate slopes over the two sets of data on *in vivo* regressions were statistically non-significant. This confirms that a single slope is applicable in each instance, which supports the assumption that the datasets come from the same population. The improved prediction equation for MT is expressed as:

$Y_{i\ \text{all samples}} = 56.12 + 0.92(x1_i)$ (values from Table 3)

The same population assumption is valid for the PME method when analysing the complete dataset. The improved prediction equation for the PME is expressed as:

$Y_{i\ \text{all samples}} = 160.6 + 0.75(x1_i)$ (values from Table 3).

Table 4 Linear regression with qualitative variable included in the model: $y_i = \beta_0 + \beta_1 x1_i + \beta_2 x2_i + \beta_3\ x1_i\ x2_i + e_i$ Same population assumption verification in the improved prediction of *in vivo* organic matter digestibility of combined feeds using modified *in vitro* and pepsin-acid multi-enzymatic methods

Method ($x1_i$)	Regr. F prob	Parameter estimates; t-probabilities, significance					n
		Intercept β_0	Slope β_1	Separate intercept[1] β_2	Separate slope[2] β_3	R^2	
		**	***	ns	Ns		
MT[3]	<0.0001	112.3036	0.8428	-73.6858	0.1065	0.942	65
		<0.0153	<0.0001	0.1613	0.1222		
		**	***	ns			
MT	<0.0001	51.89	0.92	6.57	effect excluded	0.939	68
		0.03	<0.0001	0.47			
		***	***	ns	Ns		
PME[4]	<0.0001	218.46	0.70	-92.59	0,08	0.925	65
		<0.0001	<0.0001	0,081	0,20		
		***	***	**			
PME	<0.0001	172.21	0.76	-30.53	effect excluded	0.923	68
		<0.0001	<0.0001	0.004			
MT residual analysis		Normality: Shapiro-Wilks[2] (Prob. (W=0.99) = 0.91); Kolmogorov-Smirnov (Prob. (D=0.7) > 0.15); Cramer von Mises (Prob. (W-Sq=0.42) > 0.25) Homogeneity: White' test (Prob. (Chi-sq =6.01) = 0.31)					
PME residual analysis		Normality: Shapiro-Wilks[2] (Prob.(W=0.99) = 0.91); Kolmogorov-Smirnov (Prob. (D=0.7) > 0.15); Cramer von Mises (Prob. (W-Sq = 0.42) > 0.25) Homogeneity: White's test (Prob. (Chi-sq =8.55) = 0.13)					

[1] root mean square error; 'Regr. F prob' refers to the probability associated with the overall regression F-statistic; 'separate slope' refers to separate slope-effect; 'separate intercept' refers to separate intercept-effect
ns: represents non significance; *represents 5% level of significance; **represents 1% level of significance
***represents 0.1% level of significance
[2] Shapiro-Wilks indicates normally distributed residuals, which is confirmed by normal probability plot (not included)
[3] modified Tilley & Terry (MT), [4] pepsin-acid multi-enzymatic (PME) method

The separate feedstuffs datasets and the complete diets datasets were also analysed to verify the same population assumption for the two instances (Table 5). It could be concluded from the results in Table 5 that the effect of separate slopes for 'old' and 'new' data for complete diets and was statistically non-significant. Thus, a single slope was applicable in both instances and the same population assumption was verified for both types of datasets.

The improved prediction equation for feedstuffs OMD can be stated as follows:

$Y_{i\ MT\ all\ samples} = 49.86 + 0.94(x1_i)$ (values from Table 3)
$Y_{i\ PME\ all\ samples} = 132.26 + 0.80(x1_i)$ (values from Table 3)

The prediction equations of the first dataset of complete feeds (Table 1) did not predict the second dataset accurately (Table 2) and the small sample available in the first experiment, $n_1 = 6$) when considering the R^2 values, especially for the MT, which led to an unsatisfactory validation. From Table 3 it can be stated that the combined dataset (n = 30) resulted in an improved regression equation for complete feeds. This new improved equation should be tested against a new dataset to validate it.

The improved prediction equation for complete diet OMD can be stated as follows:

$Y_{i\ MT} = 8.80 + 0.97(x1_i)$ (values from Table 3)
$Y_{i\ PME} = 224.30 + 0.66(x1_i)$ (values from Table 3)

Homogeneity for β_1 was established and proved not to be different. $\beta_1 = 1$ was determined and for MT this was true and did not differ significantly from 1, as the values were within the 95% confidence intervals.

But for PME, $\beta_1 \neq 1$ was true, and differed significantly from 1 because the upper limit of the confidence interval did not reach 1. β_1 fr MT and PME were both below 1 in real terms and may indicate an underestimation in determining apparent digestibility *in vivo*. This could be due to using only rumen flora for the MT method and only three types of enzymes and a pepsin for PME. In this study $\beta_1 = 1$ was significantly so for the MT method and underlines its importance as a method of reference, despite some reservations (Tagliapietra *et al*., 2011; Gosselink *et al*., 2004). For PME, $\beta_1 \neq 1$ indicates an underestimation of digestibility values, although when forced through 0 (no intercept) β_1 became very close to 1, but not significantly. Givens *et al*. (1990) and Aufrère & Michalet-Doreau (1988) believed that pepsin cellulase methods had a considerable role to play, as they were more accurate than chemical methods.

Table 5 Linear regression with qualitative variable included in the model: $y_i = \beta_0 + \beta_1 x1_i + \beta_2 x2_i + \beta_3\ x1_i\ x2_i + e_i$ Same population assumption verification in the improved prediction of *in vivo* organic matter digestibility of feedstuffs and complete diets using modified *in vitro* Tilly & Terry and pepsin-acid multi-enzymatic methods

Method ($x1_i$)	Regr. F prob	Parameter estimates; t-probabilities, significance				R^2	n
		Intercept β_0	Slope β_1	Separate intercept[1] β_2	Separate slope[2] β_3		
		**	***	**	**		
MT	<0.0001	154.19	0.8036	-149.05	0.21	0.96	30
		0.003	<0.0001	0.01	0.008		
		ns	***	ns			
MT	<0.0001	45.60	0.95	3.57	effect excluded	0.95	38
		0.15	<0.0001	0.79			
		***	***	**	**		
PME	<0.0001	249.43	0.67	-157.80	0.17	0.94	30
		<0.0001	<0.0001	0.01	0.04		
		***	***	**			
PME	<0.0001	163.43	0.79	-34.02	effect excluded	0.93	38
		<0.0001	<0.0001	0.04			
Complete diets: MT residual analysis		Normality: Shapiro-Wilks (Prob. (W=0.95) = 0.33); Kolmogorov-Smirnov (Prob. (D=0.12) > 0.15); Cramer von Mises (Prob. (W-Sq=0.07) > 0.25) Homogeneity: White' test (Prob. (Chi-sq =5.11) = 0.40)					
Complete diets: PME residual analysis		Normality: Shapiro-Wilks (Prob. (W=0.97) = 0.45); Kolmogorov-Smirnov (Prob. (D=0.08) > 0.15); Cramer von Mises (Prob. (W-Sq=0.03) > 0.25) Homogeneity: White's test (Prob. (Chi-sq =8.57) = 0.13) All tests indicate normality & homogeneity					
Feedstuff: MT residual analyses		Normality: Shapiro-Wilks (Prob. (W=0.98) = 0.85); Kolmogorov-Smirnov (Prob. (D=0.08) > 0.15); Cramer von Mises (Prob. (W-Sq=0.03) > 0.25) Homogeneity: White' test (Prob. (Chi-sq =3.40) = 0.64)					
Feedstuff: PME residuals analyses		Normality: Shapiro-Wilks (Prob. (W=0.99) = 0.92); Kolmogorov-Smirnov (Prob. (D=0.07) > 0.15); Cramer von Mises (Prob. (W-Sq=0.03) > 0.25) Homogeneity: White's test (Prob. (Chi-sq =2.78) = 0.73) All tests indicate normality & homogeneity					

[1] root mean square error; 'Regr. F prob' refers to the probability associated with the overall regression F-statistic 'separate slope' refers to separate slope-effect; 'separate intercept' refers to separate intercept-effect
ns: represents non significance; *represents 5% level of significance; **represents 1% level of significance ***represents 0.1% level of significance; [2] Shapiro-Wilks indicates normally distributed residuals, which is confirmed by normal probability plot (not included); [3] modified Tilley & Terry (MT); 4 pepsin-acid multi-enzymatic (PME) method

The question whether the two datasets for complete diets came from the same population could not be investigated properly because of the inability to get to a reliable prediction equation, as seen by the low R^2 value (Table 1 and Table 2) for the MT method in particular.

The use of an enzyme-based method for predicting the digestibility of feedstuffs such as maize silage (Givens *et al*., 1995), perennial ryegrass (Beecher *et al*., 2015) and legumes (Gierus *et al*., 2016) is seen as an improvement over methods that measure fibre. De Boever *et al*. (1988) found reduced accuracy of the

cellulase technique. Lower accuracy was also observed by Givens *et al*. (1995). A single equation for a forage group was proposed by De Boever *et al*. (1988) and for preparation methods by Beecher *et al*. (2015), who based this on the chemical reality that unlike a bacteria population, an enzyme cannot adapt to the available cell wall constituents. But Givens *et al*. (1995) and De Boever *et al*. (1993) showed that substantial variations in starch and cell wall contents in the various silage samples did not relate to differences in digestibilities. Regression equations based on a meganistic approach for metabolizable energy determination also showed low R^2 values (<0.73, P >0.05) (Magalhães *et al*., 2010). Beecher *et al*. (2015), Hippenstiel *et al*. (2015) and Gierus *et al*. (2016) reported R^2d values that were not higher than 0.70 for single feedstuff evaluation on OMD with one or two enzymatic treatments. This was in contrast to the high R^2d values presented in this study. There was considerable improvement and validation, leading to a more accurate prediction equation presented by the PME, which could be of great value in the routine analysis for predicting OMD.

Conclusion

Based on the non-significance of the effects included in the regression models and the amount of variation in the data, which was explained by the regression models as reflected in the R^2 values, it could be deduced that the two samples came from the same population for both the MT and the PME methods. This was true of the combined dataset and the feedstuff dataset. The improved *in vivo* prediction equation in each instance are thus valid and a true improvement on the first initial prediction equations. The R^2 values of 0.82 and 0.90, which were obtained with the available data, are sound indicators that future validation and improved prediction equations for the MT and PME methods are attainable.

The results show that a single equation to predict *in vivo* OMD by using a multi-enzymatic approach that correlates significantly with an existing MT method could be used for feedstuffs and complete diets.

Authors' Contributions

KJL participated in *in vitro* work, statistical analysis, articles and report writing, DP project initialization and University of Wageningen contact and article writing and was co-supervisor of the D-Tech student. FKS took part in article writing and supervised the D-Tech student. HM participated in statistical analysis and VAH on feeding trials at University of Wageningen.

Conflict of Interest Declaration

The authors declare that they have no competing interests.

References

AOAC, 2002. Official methods of analysis. 17th edition. Association of Official Analytical Chemists, Arlington, Virginia, USA.

Aufrère, J. & Michalet-Doreau, 1988. Comparison of methods for predicting digestibility of feeds. Anim. Feed Sci. Technol. 20, 203-218.

Beecher, M., Baumont, R., Aufrère, J., Boland, T.M., Donovan, M.O., Galvin, N., Fleming, C. & Lewis, E., 2015. A comparison of two enzymatic in vitro methods to predict in vivo organic matter digestibility of perennial ryegrass. Livest. Sci. 117, 33-42.

De Boever, J.L., Cottyn, B.G., Andries, J.I., Buysse, F.X. & Vanacker, J.M., 1988. The use of a cellulase technique to predict digestibility, metabolizable and net energy of forages. Anim. Feed Sci. Technol. 19, 247-260.

De Boever, J.L., De Brabander, D.L., De Smet, A.M., Vanacker, J.M. & Boucqué, Ch.V., 1993. Evaluation of physical structure. 2. Maize silage. J. Dairy Sci. 76, 1624-1634.

Chenost, M., Aufrère, J. & Macheboeuf, D., 2001. The gas-test technique as a tool for predicting the energetic value of forage plants. Anim. Res. 50, 349-364.

Dowman, M.G. & Collins, F.C., 1982. The use of enzymes to predict the digestibility of animal feeds. J. Sci. Food Agric. 33, 689-696.

Engels, E.A.N. & Van der Merwe, F.J., 1967. Application of an *in vitro* technique to South African forages with special reference to the effect of certain factors on the results. S. Afr. J. Agric. Sci. 10, 983-995.

Gierus, M., Eickler, B., Resh, R., Loges, R., Taube, F. & Poetsch, E.M., 2016. Using in vitro methods to estimate metabolizable energy content of five forage legumes harvested under different defoliation systems. Die Bodenkultuur, J. Land Manag. Food Environ. 67 (3), 121-131.

Givens, D.I., Dewey, P.J.S., Donaldson, E., Jones, D.I.H. & Adamson, A.H., 1990. Within- and between-centre variability in the measurement of organic matter digestibility in vivo of cereal straws. Anim. Feed Sci. Technol. 28, 103-108.

Givens, D.I., Cottyn, B.G., Dewey, P.J.S. & Steg, A., 1995. A comparison of the neutral detergent-cellulase method with other laboratory methods for predicting the digestibility in vivo of maize silages from three European countries. Anim. Feed Sci. Technol. 54, 55-64.

Gosselink, J.M.J., Dulphy, J.P., Poncet, C., Jailler, M., Tamminga, S. & Cone, J.W., 2004. Prediction of forage digestibility in ruminants using in situ and in vitro techniques. Anim. Feed Sci. Technol. 115, 227-246.

Hippenstiel, F., Kivitz, A., Benninghoff, J. & Südekum, K-H., 2015. Research note: Estimation of intestinal protein digestibility of protein supplements for ruminants using a three-step enzymatic in vitro procedure. Arch. Anim. Nutr. 69 (4), 310-318.

Hvelplund, T., Weisbjerg, M.R. & Søegaard, K., 1990. Use of *in vitro* digestibility methods to estimate *in vivo* digestibility of straws. In: Proc. TSAP Sci. Conf. Arusha, Tanzania, 3–4 August 26, 70-79.

Jones, D.I.H. & Theodorou, M.K., 2000. Enzyme techniques for estimating digestibility. In: D.I. Givens, E. Owens, R.F.E Axford & H.M. Omed (eds). Forage Evaluation in Ruminant Nutrition. CAB, New York, 2000.

Khazaal, K., Dentinho, M.T., Ribeiro, J.M. & Ørskov, E.R., 1993. A comparison of gas production during incubation with rumen contents in vitro and nylon bag degradability as predictors of the apparent digestibility in vivo and the voluntary intake. Anim. Prod. 57, 105-112

Magalhães, K.A., Valadares Filho, S.C., Detman, E., Diniz, L.L., Pina, D.S., Azevedo, J.A.G., Araújo, F.L., Marcondes, M.I., Fonseca, M.A. & Tedeschi, L.O., 2010. Evaluation of indirect methods to estimate the nutritional value of tropical feeds for ruminants. Anim. Feed Sci. Technol. 155, 44-54.

SAS/STAT User's Guide. Version 8, SAS Institute Inc., Cary, NC, USA. ISBN: 1-58025-494-2, 1999

Stern, M.D., Bach, A. & Calsamiglia, S., 1997. Alternative techniques for measuring nutrient digestion in ruminants. J. Anim. Sci. 75, 2256-2276

Tagliapietra, F., Cattania, M., Hansenb, H.H., Hindrichsenc, I.K., Bailonia, L. & Schiavon, S., 2011. Metabolizable energy content of feeds based on 24 or 48 h in situ NDF digestibility and on in vitro 24 h gas production methods. Anim. Feed Sci. Technol. 170, 182-191.

Tilley, J.M.A. & Terry, R.A., 1963. A two stage method for the in vitro digestion of forage crops. J. Br. Grassl. Soc. 18, 104-109.

Weisbjerg, R. & Hvelplund, T., 1993. Estimation of net energy content in feeds for cattle. Report No 3/1993, National Institute of Animal Science, Denmark.