Animal Feed Science and Technology 198 (2014) 49–56

Contents lists available at ScienceDirect

Animal Feed Science and Technology

journal homepage: www.elsevier.com/locate/anifeedsci

The use of cellulase and filter bag technique to predict digestibility of forages

Z.M. Kowalski[a,*], J. Ludwin[a], P. Górka[a], M. Rinne[b], M.R. Weisbjerg[c], W. Jagusiak[d]

[a] University of Agriculture in Krakow, Department of Animal Nutrition and Dietetics, Al. Mickiewicza 24/28, 30-059 Krakow, Poland
[b] MTT Agrifood Research Finland, Animal Production Research, FI-31600 Jokioinen, Finland
[c] Department of Animal Science, Aarhus University, AU Foulum, PO Box 50, DK 8830 Tjele, Denmark
[d] University of Agriculture in Krakow, Department of Genetics and Animal Breeding, Al. Mickiewicza 24/28, 30-059 Krakow, Poland

ARTICLE INFO

Article history:
Received 24 March 2014
Received in revised form
14 September 2014
Accepted 15 September 2014

Keywords:
In vitro
Prediction
Organic matter digestibility
Forage
ANKOM Daisy[II] Incubator

ABSTRACT

A study was conducted to determine the reliability of the novel *in vitro* dry matter digestibility (IVDMD) method for predicting the *in vivo* organic matter digestibility (OMD) of forages. The study was carried out on two sets of feeds of known OMD determined on sheep: set A ($n = 35$), consisting of whole crop cereal (corn and barley) herbages and set B ($n = 80$), mostly consisting of grass and clover silages. IVDMD was determined by the CEL48-ND method in which 500 mg of dried and ground feed sample was inserted into filter bag (ANKOM Technology Corporation, Fairport, NY, USA) and then the bags were incubated for 48 h at 39 °C in the cellulase solution (celullase Onozuka R10), in the jars of Daisy[II] Incubator (ANKOM Technology Corporation). After incubation the bags with residues were extracted in a neutral detergent (ND) for 1 h at 100 °C in Ankom[220] Fiber Analyzer (ANKOM Technology Corporation). The data for all sets were used to find the best single and multiple regression equations to predict OMD. For each set the linear regressions were calculated in which OMD was the dependent variable (Y) whereas IVDMD determined by CEL48-ND was the predictor X_1 and contents of nutrients (g/kg DM) were predictors X_2, X_3, X_4 or X_5. The equations were compared by means of coefficient of determination (R^2), standard deviation of differences between observed (Y_i) and predicted value ($\tilde{Y}_i$) (residual standard deviation; RSD), and mean square prediction error (MSPE). Additionally, the verification of equations was also done based on the Akaike information criterion (AIC). Mean R^2 for the regression equations of OMD by CEL48-ND was 0.468 (set A), 0.581 (set B) and 0.539 (set A + B). Including the next predictor (X_2, X_3, X_4 or X_5) increased R^2, and decreased RSD, MSPE and AIC, especially in the equations calculated for whole crop corn herbages, grass silages and clover silages. It can be concluded that the *in vitro* method presented in this study is a simple alternative for existing methods in which buffered rumen fluid is used. Using a standard enzyme available commercially worldwide may decrease variation between laboratories. Further, using filter bags and Daisy Incubator decreases labour costs and use of animals.

© 2014 Elsevier B.V. All rights reserved.

Abbreviations: AIC, akaike information criterion; ADF, acid detergent fibre; aNDF, neutral detergent fibre; AFBT, ANKOM filter bag technique; CP, crude protein; CS, subset 'clover silages'; DK, Denmark; DM, dry matter; FIN, Finland; GS, subset 'grass silages'; IVDMD, *in vitro* dry matter digestibility; MSPE, mean square prediction error; ND, neutral detergent solution; OMD, organic matter digestibility; R^2, coefficient of determination; RSD, residual standard deviation; WCB, subset 'whole crop barley'; WCC, subset 'whole crop corn'.

* Corresponding author at: Department of Animal Nutrition and Dietetics, University of Agriculture in Krakow, Al. Mickiewicza 24/28, 30-059 Krakow, Poland. Tel.: +48 12 662 40 84; fax: +48 12 633 62 45.
E-mail address: rzkowals@cyf-kr.edu.pl (Z.M. Kowalski).

http://dx.doi.org/10.1016/j.anifeedsci.2014.09.008
0377-8401/© 2014 Elsevier B.V. All rights reserved.

1. Introduction

High demands for nutrients of modern dairy cows and fast growing beef cattle require a precise diet formulation, based on a reliable evaluation of the nutritive value of feedstuffs, especially forages. While the accuracy of determination of chemical composition is mostly not questionable, digestibility values are still the weakest link in estimation of forage nutritive value. *In vivo* digestibility methods are costly, laborious and inaccessible for most field laboratories, as well as they increasingly raise the concerns about animal welfare (Adesogan, 2002; Huhtanen et al., 2006). On the other hand, majority of the most popular *in vitro* methods (*e.g.* Tilley-Terry or gas-test) requires buffered rumen fluid as an incubation medium (Tilley and Terry, 1963; Goering and Van Soest, 1970; Menke and Steingass, 1988; Hall and Mertens, 2012) which is a serious limitation for laboratories not having an access to cannulated animals to donate the rumen fluid. Such methods are also difficult to standardize due to different feeding conditions of donor animals in different laboratories (Adesogan, 2002; Hall and Mertens, 2012).

Above discussed limitations with an access to rumen fluid encourage the use of enzyme solutions as incubation medium instead of rumen fluid (Jones and Theodorou, 2000; Huhtanen et al., 2006). Among few, the fungal cellulase-based techniques have been the most often tested (De Boever et al., 1988; Aufrère and Graviou, 1996; Adesogan, 2002; Nousiainen et al., 2003). According to Huhtanen et al. (2006), the enzymatic hydrolysis reflects the mechanism of digestibility better than concentrations of components from proximate analysis or detergent fractionation. However, the calibration of enzyme digestion assays has been a major limitation in practical adaptation of them (Broderick and Colombini, 2010). Moreover, enzyme-based predictions of *in vivo* digestibility also vary with forage species, population and season of harvest (Givens et al., 1995; Huhtanen et al., 2006). Thus, forage-specific equations may be needed. Although the *in vitro* digestibility estimated using enzymatic techniques is often lower than *in vivo* digestibility, these differences do not preclude the use of such methods provided that appropriate correction equations are used (Huhtanen et al., 2006).

An important source of variation in the results of *in vitro* gravimetric methods is related to the filtering step (Adesogan, 2002). Enclosing the sample in the filter bag, such as in the method developed by ANKOM Technology Corporation (Fairport, NY, USA), may eliminate this problem. In ANKOM filter bag technique (AFBT), feed samples sealed in the polyester bags are placed in glass jars which are rotated in an insulated chamber, called the Daisy^{II} Incubator. The AFBT also reduces the labour input because it allows batch incubation of several samples in the jar (Adesogan, 2002). The original AFBT is based on the '*in vitro* true digestibility' method described by Goering and Van Soest (1970). It was shown in several studies that digestibility estimated by AFBT correlated well with conventional *in vitro* techniques, *e.g.* Tilley-Terry (Holden, 1999; Mabjeesh et al., 2000; Wilman and Adesogan, 2000; Adesogan, 2005; Ammar et al., 2005). On the other hand, for some forages Vogel et al. (1999) showed higher dry matter (DM) digestibility estimated by AFTB than by the conventional *in vitro* technique. Furthermore, in a study of Damiran et al. (2008) digestibility values estimated by AFBT were correlated (R^2 = 0.58–0.88) with values estimated by conventional *in vitro* techniques, but in most cases AFBT overestimated *in vivo* DM and NDF digestibility. The study of Damiran et al. (2008) is one of the few that attempted to correlate AFBT with *in vivo* observations. According to Wilman and Adesogan (2000), the conventional *in vitro* technique is likely to give more precise results than AFBT, although they postulated that the use of AFBT gave acceptable digestibility estimates for forages when the emphasis was on saving labour.

To our best knowledge only one study has been published in which fibrolytic enzyme mixtures (cellulase and hemicellulase) were used instead of rumen fluid in the Ankom Daisy^{II} Incubator (Colombatto et al., 2000). The study showed that the enzyme mixtures had the potential to describe the DM digestibility of forages.

The present study was conducted to determine the reliability of the novel *in vitro* DM digestibility (IVDMD) method using cellulase and filter bag technique for predicting the *in vivo* organic matter digestibility (OMD).

2. Materials and methods

2.1. Feeds and chemical analyses

The study was carried out on two sets of feeds. Set A (n = 35) consisted of two subsets: "whole crop corn; WCC" (n = 20) consisting of whole crop corn silages (n = 10) and whole crop corn herbages (n = 10) (WCC) as well as "whole crop barley; WCB" (n = 15) consisting of whole crop barley silage (n = 10) and whole crop barley herbages (n = 5). Ensiled and un-ensiled samples were not originated from same source.

Set B (n = 80), consisting of two subsets: "grass silage; GS" (n = 54) consisting of timothy-meadow fescue grass silages as well as "clover silage; CS" (n = 19) consisting of red clover silages. Additionally in set B there were whole crop barley silages (n = 5) and whole crop wheat silages (n = 2).

Samples of feeds of set A originated from Denmark (DK; Department of Animal Science, Aarhus University, AU Foulum), whereas samples of set B originated from Finland (FIN; MTT Agrifood Research Finland, Animal Production Research). Original feeds were dried in force-air oven at 50 (DK) or 60 °C (FIN) for until dry and dried samples were ground to pass 1 mm screen.

Chemical composition was determined by standard methods (AOAC, 2000), using the following procedures: DM–930.15, ash–923.03, crude protein (CP)–990.03, ether extract–920.39. Neutral detergent fibre (aNDF) was determined with heat-stable amylase according to Van Soest et al. (1991). Acid detergent fibre (ADF) and lignin (sa) were determined using the method described by Robertson and Van Soest (1981). Above fibre analysis were performed using Ankom^{220} Fiber Analyzer.

Cellulose content was calculated as ADF–lignin, whereas hemicelluloses as aNDF–ADF. Starch in feeds (only set A) was determined by the enzymatic method of Faisant et al. (1995).

2.2. In vivo digestibility

In vivo OMD was determined using total faecal collection method. The evaluations were carried out on 6 (DK) or 4 (FIN) adult rams fed near the maintenance level and with free access to water. For set B, forage was fed as the sole ration supplemented with mineral-vitamin mixture and salt. For set A, forages were low in crude protein, and were supplemented with soybean meal with known digestibility, as well as with mineral-vitamin mixture and salt, and digestibility of forages was calculated using the difference method. The Danish samples of WCC and WCB subset samples were frozen in daily meals portions and thawed before feeding. For each feed, the study period consisted of 7 days of preliminary period and 7 days for total faecal collection.

2.3. In vitro digestibility

IVDMD was determined by the CEL48-ND method. Approximately 500 mg of each sample (dried and ground as described above) was inserted into filter bag (F57; 50 × 55 mm; ANKOM Technology Corporation, Fairport, NY, USA), which was then heat-sealed. Before weighing out the bags were soaked in acetone for ca. 5 min and dried to constant weight.

The bags filled with samples were incubated in the jars of Daisy^{II} Incubator (ANKOM Technology Corporation, Fairport, NY, USA) for 48 h at 39 °C. The Daisy^{II} rotated the jars continuously in the incubation chamber. In each of the four jars there were 25 bags (6 replicates × 4 feeds + 1 blank). The incubation inoculum was a preheated (at 39 °C) cellulase solution (celullase Onozuka R10, *Trichoderma viride*; Medicine Department, Yakult Honskha LTD, Tokyo, Japan; 1 IU/mg, EC 3.2.1.4., Merck, Germany). The concentration of cellulase in the solution was 1 g per 1 L of 0.01 M acetate buffer (pH 4.6).

After incubation, the jars were drained and the bags rinsed thoroughly with tap water followed by an extraction with a neutral detergent solution (ND; Goering and Van Soest, 1970) for 1 h at 100 °C, in Ankom^{220} Fiber Analyzer (ANKOM Technology Corporation, Fairport, NY, USA). The bags with residue were again rinsed gently in cold water, dried in a forced-air oven for 48 h at 60 °C and weighed.

Additionally, for samples from the set A, the IVDMD was determined by the RF48-ND method, with rumen fluid as the incubation inoculum. The procedures of sample and bag preparation were the same as for the CEL48-ND method. The bags with samples were incubated in buffered rumen fluid for 48 h at 39 °C. Rumen digesta was collected before morning feeding from two dry Holstein cows fitted with a ruminal cannula. The animals were fed standard diets to meet their maintenance requirements (5 kg meadow hay, 2 kg ground barley and 100 g mineral-vitamin supplement).

After purging with CO_2, the digesta from both cows was transported to the laboratory in an insulated bottle (for 30 min), filtered through four layers of cheese cloth, mixed, again purged with CO_2, mixed with the buffer and warmed to 39 °C. Finally RF was poured to the jars of Daisy^{II} incubator, previously filled with warmed (39 °C) mixture of solution A and B (v/v as 1:5; pH 6.8). The reagents used were: solution A (in 1 L of deionized water)–KH_2PO_4 (10.0 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), NaCl (0.5 g), $CaCl_2 \cdot 2H_2O$ (0.1 g) and urea (0.5 g); solution B (in 100 mL of deionized water)–Na_2CO_3 (15.0 g) and $Na_2S \cdot 9H_2O$ (1.0 g) (Holden, 1999). After mixing and purging with CO_2 for 1 min, the inoculum was again warmed to 39 °C. The procedure of incubation of bags was the same as in the CEL48-ND method, including an extraction of the residue with ND.

In both methods, the IVDMD was calculated as the DM which disappeared from the initial weight and the final result was the mean of 6 replicates.

2.4. Calculations and statistical analysis

The differences between IVDMD determined by CEL48-ND and RF48-ND for set A and its subsets were statistically tested by one-way analysis of variance using the GLM procedure of SAS (2008).

Using the procedure REG of SAS (2008), the data for all sets and subsets were used to find the best single and multiple regression equations to predict *in vivo* OMD. For each set and subset the linear regressions were calculated in which *in vivo* OMD was the dependent variable (Y) whereas IVDMD determined by CEL48-ND was the predictor X_1 and contents of nutrients (g/kg DM) were predictors X_2, X_3, X_4 or X_5. The equations were compared by means of coefficient of determination (R^2), standard deviation of differences between observed (Y_i) and predicted value ($\tilde{Y}_i$) (residual standard deviation; RSD), and mean square prediction error (MSPE), calculated according to Bibby and Toutenberg (1977). Additionally, the verification of equations was also done based on the Akaike information criterion (AIC) for each set or subset (SAS, 2008).

For the whole dataset (sets A and B) in order to avoid the overfitting and improve predictive performance of the multiple regression model the excessive number of parameters was reduced and only suitable independent variables were proposed. The final model was set using the stepwise selection at the significance level of 0.15 (SAS, 2008).

Table 1
Chemical composition (g/kg dry matter), *in vivo* organic matter digestibility (OMD) and *in vitro* dry matter digestibility determined by a cellulase (CEL48-ND) or rumen fluid (RF48-ND) based methods; samples from set A and subsets "whole crop corn" and "whole crop barley".

Items	Organic matter	Crude protein	Ether extract	aNDF	ADF	Lignin	Cellulose	Hemicellulose	Starch	OMD	CEL48-ND[a]	RF48-ND[b]
Set A (n = 35)												
Mean[c]	956	91	32	434	249	25	224	184	272	0.720	0.710	0.679
Standard deviation	8	14	9	37	28	15	29	28	61	0.039	0.039	0.033
Minimum	933	65	20	357	206	6	169	128	115	0.632	0.641	0.603
Maximum	968	124	60	505	317	61	290	265	355	0.785	0.842	0.746
Median	957	92	31	425	248	21	229	187	278	0.718	0.710	0.670
Subset "whole crop corn" (WCC; n = 20)[d]												
Mean[c]	956	89	32	427	250	23	227	176	290	0.717	0.711	0.681
Standard deviation	71	14	7	38	34	13	32	26	57	0.044	0.044	0.031
Subset "whole crop barley" (WCB; n = 15)[e]												
Mean[c]	956	92	32	443	248	28	220	195	249	0.726	0.709	0.676
Standard deviation	10	14	11	36	17	17	25	28	59	0.033	0.032	0.037

[a] CEL48-ND–incubation in cellulase for 48 h, followed by extraction in neutral detergent solution.
[b] RF48-ND–incubation in rumen fluid for 48 h, followed by extraction in neutral detergent solution.
[c] Significant differences between CEL48-ND and RF48-ND (P<0.001).
[d] Subset "whole crop corn" consisting of whole crop corn silages and whole crop corn herbages.
[e] Subset "whole crop barley" consisting of whole crop barley silages and whole crop barley herbages.

3. Results

The large range of variation in chemical composition and *in vivo* OMD (0.632-0.785) of feeds from set A made it a suitable database for evaluation of the CEL48-ND method (Table 1). Mean chemical composition and *in vivo* OMD of feeds of subset WCC were similar to subset WCB, except for a much higher content of starch and lower lignin in WCC.

The IVDMD determined by CEL48-ND was significantly higher than that determined by the RF48-ND for set A and both WCC and WCB subsets (Table 1; P<0.001). Mean IVDMD determined by the CEL48-ND was lower than *in vivo* OMD, regardless of set or subsets, whereas IVDMD values determined by the RF48-ND were even lower than CEL48-ND ones.

Mean R^2 for the regression equations of *in vivo* OMD by CEL48-ND was 0.468, 0.698 and 0.135 for set A, subsets WCC and WBC, respectively (Table 2; equations 1, 4 and 7). The regression equations with 1, 2 or 3 independent variables used to predict *in vivo* OMD for feeds based on set A and subsets WCC and WCB are presented in Table 2. In each equation, the IVDMD determined by CEL48-ND was predictor X_1. Other predictors used in the equations (X_2 or X_3) were for the set A the contents of ash, ADF or hemicellulose (% in DM). In all cases each additional variable in the equation increased R^2. The R^2 of equations for subset WCC were higher than those for set A, whereas R^2 for subset WCB were substantially lower. Equations for WCB contained the contents of CP and ash. For each equation for set A, the RSD were less than 2.90 (Table 2). Including the second (X_2) and third predictor (X_2 and X_3) decreased RSD, MSPE and AIC. Regression coefficient for CEL48-ND in equation 2 was not significantly different from zero.

The RSD, MSPE and AIC of equations for subset WCC were much lower than those for whole set A. Additional one (X_2) or two predictors (X_2 and X_3) decreased RSD, MSPE and AIC. In all equations for WCC the coefficients of regression for CEL48-ND were significant. The RSD and MSPE for equations calculated for WCB were much higher than those for the set A and subset

Table 2
Regression equations for estimation of *in vivo* organic matter digestibility (Y); set A and subsets "whole crop corn" and "whole crop barley".

Number of equation	Equation[a]	R^2[b]	RSD[c]	MSPE[d]	AIC[e]
Set A (n = 35)					
1	Y = 0.688 CEL48-ND + 0.232	0.468	2.89	7.87	74.84
2	Y = 0.252 CEL48-ND – 0.0008 ADF + 0.732[f]	0.568	2.63	6.34	69.76
3	Y = 0.908 CEL48-ND + 0.0016 Ash + 0.0006 Hemicellulose – 0.111	0.639	2.45	5.30	65.65
Subset "whole crop corn" (WCC; n = 20)					
4	Y = 0.821 CEL48-ND + 0.133	0.698	2.53	5.79	37.05
5	Y = 0.947 CEL48-ND + 0.0008 Hemicellulose – 0.106	0.930	1.22	1.26	11.19
6	Y = 1.063 CEL48-ND + 0.0009 Hemicellulose + 0.0012 Ash – 0.261	0.955	1.01	0.81	4.90
Subset "whole crop barley" (WCB; n = 15)					
7	Y = 0.371 CEL48-ND + 0.462[f]	0.135	3.16	8.65	36.38
8	Y = 0.495 CEL48-ND + 0.0008 CP + 0.296[f]	0.263	3.04	7.38	35.98
9	Y = 0.551 CEL48-ND + 0.0006 Ash + 0.0008 CP + 0.238[f]	0.288	3.12	7.13	37.47

[a] Constraints X_1 –*in vitro* dry matter digestibility determined by CEL48-ND method (%; CEL48-ND), X_2, X_3–content of selected nutrients (% dry matter).
[b] Coefficient of determination.
[c] Residual standard deviation.
[d] Mean square prediction error.
[e] Akaike information criterion.
[f] Regression coefficient for CEL48-ND not significant.

Table 3
Chemical composition (g/kg dry matter), *in vivo* organic matter digestibility (OMD), and *in vitro* dry matter digestibility determined by a cellulase based method (CEL48-ND); samples from set B and subsets "grass silage" and "red clover silage", and whole crop silage of barley and wheat.

Items	Organic matter	Crude protein	Ether extract	aNDF	ADF	Lignin	Cellulose	Hemicellulose	OMD	CEL48-ND
Set B (n = 80)										
Mean	914	157	38	496	289	32	256	207	0.712	0.665
Standard deviation	15	36	11	105	58	12	53	57	0.055	0.076
Minimum	878	107	16	274	153	17	134	90	0.581	0.510
Maximum	940	256	50	669	390	79	338	282	0.840	0.835
Median	915	151	40	510	291	30	265	221	0.711	0.656
Subset "grass silage" (GS; n = 54)[a]										
Mean	918	150	47	553	315	31	284	238	0.716	0.644
Standard deviation	13	29	7	61	37	8	30	28	0.056	0.071
Subset "red clover silage" (CS; n = 19)[b]										
Mean	898	193	32	356	239	39	200	117	0.710	0.730
Standard deviation	11	30	11	65	59	17	44	17	0.055	0.063
Whole crop barley silages (n = 5)										
Mean	932	111	21	416	205	24	182	210	0.703	0.662
Standard deviation	6	3	4	68	47	7	40	22	0.045	0.059
Whole crop wheat silages (n = 2)										
Mean	913	124	18	472	261	35	226	211	0.643	0.612
Standard deviation	3	3	2	30	30	3	27	7	0.016	0.040

[a] Subset "grass silage" consisting of timothy-meadow fescue grass silages.
[b] Subset "clover silage" consisting of red clover silages.

Table 4
Regression equations for estimation of *in vivo* organic matter digestibility (Y); set B and subsets "grass silage" and "red clover silage".

Number of equation	Equation	R^2[b]	RSD[c]	MSPE[d]	AIC[e]
Set B (n = 80)					
1	Y = 0.546 CEL48-ND + 0.349	0.581	3.57	12.43	205.58
2	Y = 0.882 CEL48-ND + 0.0006 Hemicellulose − 0.001	0.817	2.38	5.43	141.38
3	Y = 0.868 CEL48-ND + 0.0003 aNDF − 0.0014 Lignin − 0.003	0.879	1.94	3.58	110.05
Subset "grass silage" (GS; n = 54)					
4	Y = 0.695 CEL48-ND + 0.268	0.787	2.59	6.46	104.70
5	Y = 1.071 CEL48-ND + 0.0011 Cellulose − 0.279	0.896	1.83	3.15	68.04
6	Y = 1.039 CEL48-ND + 0.0007 aNDF − 0.0022 Lignin − 0.296	0.916	1.66	2.54	58.27
Subset "red clover silage" (CS; n = 19)					
7	Y = 0.800 CEL48-ND + 0.126	0.820	2.41	5.21	35.36
8	Y = 0.552 CEL48-ND − 0.0012 Lignin + 0.356[a]	0.896	1.90	3.03	27.03
9	Y = 0.532 CEL48-ND − 0.0003 Hemicellulose − 0.0012 Lignin + 0.399	0.900	1.91	2.88	28.11

[a] Regression coefficient for CEL48-ND not significant.
[b] Coefficient of determination.
[c] Residual standard deviation.
[d] Mean square prediction error.
[e] Akaike information criterion.

WCC. Including the third predictor (X_3) did not change the error of prediction much. In all equations for this subset the coefficients of regression for CEL48-ND were not significant.

The range of variation in chemical composition and in *in vivo* OMD (0.581–0.840) of feeds from set B was large, making it a suitable dataset for evaluation of the CEL48-ND method (Table 3). Feeds of subsets GS and CS differed in chemical composition. Feeds in subset CS contained more CP and lignin, but less aNDF, ADF, cellulose and hemicellulose than those in GS. The subsets did not differ in mean *in vivo* OMD, but the mean IVDMD determined by CEL48-ND was much lower in subset GS.

Mean R^2 of *in vivo* OMD by CEL48-ND was 0.581, 0.787 and 0.820 for set A and subsets GS and CS, respectively (Table 4; equations 1, 4 and 7). The regression equations with 1, 2 or 3 independent variables used to predict *in vivo* OMD for feeds of set B and subsets GS and CS are presented in Table 4. The variables selected for feeds of the whole set B and subsets GS and CS were the contents of aNDF, hemicellulose, cellulose and lignin.

Including the next variable to the equation, *i.e.* X_2 or X_2 and X_3 increased R^2 for set B and its subsets. The R^2 values for both subsets were higher than for set B. Adding of a third variable did not increase R^2 substantially.

The RSD of equations calculated for set B were less than 3.60 (Table 4). In all equations the regression coefficients for CEL48-ND were significant. The RSD, MSPE and AIC for equations calculated for subset GS were much lower than those for set B. For GS, including the variable X_3 (*e.g.* lignin) improved the prediction of *in vivo* OMD. On the other hand, adding the variable X_3 did not decrease RSD, MSPE and AIC for subset CS. For this subset, the coefficient of regression for CEL48-ND for the equation with 2 variables (equation 8) was not significant.

Table 5
Chemical composition (g/kg dry matter), *in vivo* organic matter digestibility (OMD) and *in vitro* dry matter digestibility determined by a cellulase based method (CEL48-ND); samples from whole dataset including whole crop cereals forages, grass silages and red clover silages.

Items	Organic matter	Crude protein	Ether extract	aNDF	ADF	Lignin	Cellulose	Hemicellulose	OMD	CEL48-ND
Set A and B (n = 115)										
Mean	927	137	36	477	277	30	246	200	0.714	0.679
Standard deviation	24	44	10	94	53	13	49	51	0.051	0.070
Minimum	878	65	16	274	153	6	130	90	0.581	0.510
Maximum	968	256	60	669	390	79	338	282	0.840	0.835
Median	926	128	36	482	272	28	245	208	0.715	0.675

Table 6
Summary of stepwise selection of variables; whole dataset including whole crop cereals forages, grass silages and red clover silages.

Step	Variable entered	Partial R^2	Model R^2	$C(p)$[a]	F value	P[b] > F
Set A and B (n = 115)						
1.	Cel48-ND	0.539	0.539	131.94	132.21	<.0001
2.	Hemicellulose	0.209	0.749	23.51	93.35	<.0001
3.	Cellulose	0.022	0.771	13.82	10.74	0.0014
4.	CP	0.014	0.785	8.57	7.02	0.0092
5.	Lignin	0.011	0.795	4.84	5.78	0.0179

[a] $C(p)$–Mallows' Cp statistic.
[b] P–Probability.

Table 7
Regression equations for estimation of *in vivo* organic matter digestibility (Y); whole dataset including whole crop cereals forages, grass silages and red clover silages.

Number of equation	Equation	R^2[a]	RSD[b]	MSPE[c]	AIC[d]
Set A and B (n = 115)					
1	Y = 0.527 CEL48-ND + 0.356	0.539	3.44	11.66	286.46
2	Y = 0.817 CEL48-ND + 0.0006 Hemicellulose + 0.039	0.749	2.56	6.36	218.74
3	Y = 0.919 CEL48-ND + 0.0005 Hemicellulose + 0.0002 Cellulose − 0.079	0.771	2.45	5.80	210.12
4	Y = 0.883 CEL48-ND + 0.0006 Hemicellulose + 0.0002 Cellulose + 0.0001 CP − 0.070	0.785	2.39	5.45	205.00
5	Y = 0.808 CEL48-ND + 0.0005 Hemicellulose + 0.0002 Cellulose + 0.0002 CP − 0.0005 Lignin + 0.005	0.795	2.34	5.18	201.06

[a] Coefficient of determination.
[b] Residual standard deviation.
[c] Mean square prediction error.
[d] Akaike information criterion.

Chemical composition, *in vivo* OMD and IVDMD determined by CEL48-ND method of feeds from set A and B (n = 115 feeds) are presented in Table 5. The effect of each subsequent variable on the significance of a model was tested using the stepwise selection method (Table 6). The adding of a fourth or fifth variable (X_4 or X_5) had no further effect.

The R^2 for regression of *in vivo* OMD on CEL48-ND was 0.539 (Table 7; equation 1). Including contents of cellulose and hemicellulose as a second and third variable in the regression equation decreased RSD, MSPE and AIC (Table 7; equations 2 and 3). For each equation in Table 7, the coefficients of regression for CEL48-ND were significant.

4. Discussion

For obtaining the most applicable *in vitro* method of determination of feedstuff digestibility for ruminants, the following features of such methods have been studied: repeatability within laboratories and between them, reliability against *in vivo* digestibility, animal welfare, labour, cost, simplicity and possibility of using in field laboratories (Adesogan, 2002; Huhtanen et al., 2006; Hall and Mertens, 2012). The most popular *in vitro* methods, such as Tilley and Terry (1963) or the gas technique (Menke and Steingass, 1988), do not fulfil these needs, mostly due to the requirement of cannulated animals. It seems that the CEL48-ND method, tested in our study, may be an attractive alternative. The principles of the CEL48-ND method are: an enclosing of samples in the filter bags which are than incubated in the digestion vessels of Daisy^{II} Incubator, filled with the cellulase solution as incubation medium followed by the extraction of residues in ND. The enzymatic ND-cellulase method has been tested by others (Lila et al., 1986; Julier et al., 1999) but they used neither filter bags nor Daisy^{II} Incubator, and the first step in above studies was removing of cell solubles by ND followed by incubation in buffered enzyme solution. On the other hand, filter bags and Daisy^{II} Incubator were applied in several studies (Holden, 1999; Vogel et al., 1999; Mabjeesh et al., 2000; Wilman and Adesogan, 2000; Adesogan, 2005; Ammar et al., 2005; Damiran et al., 2008). However in these studies rumen fluid was used as the incubation inoculum. Colombatto et al. (2000) have probably been the only until now to study the use of enzyme mixture in AFBT.

The conditions of the CEL48-ND method were established in a study by Ludwin (2007). The source of cellulase and its concentration in the solution was as suggested by Aufrère and Graviou (1996). Neither increasing of this concentration from 1 to 2 g per 1 L nor the particle size (1 or 1.5 mm) had an effect on digestibility (Ludwin, 2007). Using of cellulase instead of rumen fluid eliminates a drawback of the AFBT based on rumen fluid. A simultaneous incubation of forage and concentrate samples (associative effect of between samples) in the same digestion vessel filled with rumen fluid may lead to errors (Adesogan, 2002). The estimated *in vitro* digestibility was called *in vitro* dry matter digestibility (Adesogan, 2005; Ammar et al., 2005). The results of the original AFBT are often called *in vitro* NDF digestibility (IVNDFD; Hall and Mertens, 2012).

The relationship between IVDMD estimated by two *in vitro* methods (CEL48-ND and RF48-ND) was close. The AFBT "original" method based on incubation of samples in buffered rumen fluid has been compared with conventional *in vitro* methods (Holden, 1999; Vogel et al., 1999; Wilman and Adesogan, 2000; Damiran et al., 2008). According to Wilman and Adesogan (2000) the traditional methods using tubes are likely to give more precise results than filter bags, although at the cost of more labour. Vogel et al. (1999) showed that the relationship between the results of AFBT and conventional *in vitro* technique depended on forage type. On the other hand, AFBT technique for most forages was more efficient in solubilising sample DM than the Tilley-Terry IVDMD (Damiran et al., 2008). The AFBT DM digestibilities were also higher than those determined *in vivo* (Damiran et al., 2008), but as *in vivo* values are apparent while AFTB present the true digestibility (*e.g.* no metabolic and endogenic DM production), the absolute values cannot be directly compared. However, digestibility values estimated by AFBT correlated well with values estimated by conventional *in vitro* and *in vivo* techniques. The above study by Damiran et al. (2008) is the only in which AFBT was validated against *in vivo* data. To our best knowledge the present study is the first one to use cellulase solution as an incubation medium as well as filter bags and Daisy Incubator.

The reliability of CEL48-ND method was tested on two sets of feeds, with known *in vivo* OMD. The feeds were the most popular forages used in ruminant nutrition in Europe, *i.e.* whole crop corn forages, whole crop barley forages, grass silages and clover silages. The number of feeds in each set and subset, as well as the variation in chemical composition and *in vivo* OMD were appropriate to test correlations. For all feeds tested (set A and B) the relationship between IVDMD estimated by CEL48-ND and *in vivo* OMD was quite close ($R^2 = 0.539$). However, this relationship differed among types of forages, being the closest for grass silages ($R^2 = 0.787$) and clover silages ($R^2 = 0.820$) and the weakest for whole crop barley ($R^2 = 0.135$).

The reason for poor relationship for whole crop barley forages, especially when compared with whole crop corn forages, is not clear. It could be a small range in digestibility values for this subset. On the other hand, it cannot be the interference of starch since whole crop corn forages contained even more starch than barley whole crop forages. However, the corn samples were much more physically homogeneous. The particle size of a sample is important for representative sampling in *in vitro* studies in which only 0.5 g sample is incubated.

For the WCC forages, the variables which influenced the digestibility most were structural carbohydrates and ash, whereas for the WCB forages the predictors were CP and ash contents. Although the CEL48-ND method seems to be more applicable for WCC than WCB, low RSD and MSPE values for the equations calculated for the whole set A may indicate that they can be used to predict *in vivo* OMD of whole crop corn and barley forages.

The CEL48-ND method may also be used to predict *in vivo* OMD of grass or clover silages, especially when two or three predictors are considered.

For the GS and CS the variables which influenced the digestibility most were structural carbohydrates and lignin. In general, the inoculum used in the *in vitro* methods (*e.g.* rumen fluid, cellulase) are not as efficient as animals in digesting of fibre, but a removal of cell solubles is easier. Because the fibre concentration was higher in grass silages than in clover silages, the feeds from subset GS had lower IVDMD, irrespective of a lack of differences between subsets in *in vivo* OMD. Such differences between subsets in IVDMD support use of separate equations for grass silages and clover silages.

The prediction of *in vivo* OMD for the whole dataset (A and B) was poorer than for subsets WCC, GS and CS. It seems that CEL48-ND method may be more useful for fibrous feeds (grasses and clover) than for whole crop cereals, especially barley. These results show that in the practical application of the CEL48-ND method, the type of forage should be considered. Also Huhtanen et al. (2006) showed a significant effect of forage type, characterized by chemical parameters and digestibility, on prediction errors of regression equations with pepsin-cellulase OM solubility as predictor and *in vivo* OMD as a dependent variable.

As shown by the stepwise selection the predictors for *in vivo* OMD were structural carbohydrates, lignin and CP. However, adding more than three independent variables seems to be useless. The equations with two or three variables (*i.e.* CEL48-ND, hemicellulose, cellulose) may have practical application for a wide range of forages.

In conclusion, the *in vitro* method presented in this study is a simple alternative for existing methods in which buffered rumen fluid is used. Using a standard enzyme available commercially worldwide may decrease variation between laboratories. Further, using filter bags and Daisy[II] Incubator decreases labour costs.

Acknowledgements

The study was financially supported by the State Committee for Scientific Research (Komitet Badan Naukowych; grant 2 P06Z 062 26).

References

Adesogan, A.T., 2002. What are feeds worth?: A critical evaluation of selected nutritive value methods. In: Proc. 13th Annual Florida Ruminant Nutrition Symposium, pp. 33–47.

Adesogan, A.T., 2005. Effect of bag type on the apparent digestibility of feeds in Ankom Daisy II Incubators. Anim. Feed Sci. Technol. 119, 333–334.

Ammar, H., Lopez, S., Gonzales, J.S., 2005. Assessment of the digestibility of some Mediterranean shrubs by in vitro techniques. J. Anim. Feed Sci. 119, 323–331.

AOAC, 2000. Official Methods of Analysis, 17th ed. Association of Official Analytical Chemists, Washington, DC.

Aufrère, J., Graviou, D., 1996. Prévision de la digestibilité des fourrages et aliments concentrés et composés des Herbivores par une méthode enzymatique pepsine-cellulase. Fourrages. BIPEA, Septembre, pp. 14.

Bibby, J., Toutenberg, H., 1977. Prediction and Improvement Estimation in Linear Models. John Wiley & Sons, London, pp. UK–UK9.

Broderick, G.A., Colombini, S., 2010. In vitro methods to determine rate and extent of ruminal protein degradation. In: Crovetto, G.M. (Ed.), Energy and Protein Metabolism and Nutrition EAAP Publication No. 127. Wageningen Academic Publishers, pp. 691–702.

Colombatto, D., Mould, F.L., Bhat, M.K., Owen, E., 2000. Fibrolytic enzyme mixtures as an alternative to rumen fluid for assessing feed degradation kinetics in vitro. Proc. Brit. Soc. Anim. Sci., 27.

Damiran, D., Del Curto, T., Bohnert, D.W., Findholt, S.L., 2008. Comparison of techniques and grinding size to estimate digestibility of forage based ruminant diets. Anim. Feed Sci. Technol. 141, 15–35.

De Boever, J.L., Cottyn, B.G., Andries, J.L., 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.

Faisant, N., Planchot, V., Kozlowski, F., Pacouret, M.P., Colonna, P., Champ, M., 1995. Resistant starch determination adapter to products containing high level of resistant starch. Sci. Alim. 15, 83–89.

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.

Goering, H.K., Van Soest, P.J., 1970. Forage Fibre Analyses (Apparatus, Reagents, Procedures, and some Applications). Agriculture Handbook No. 379. Agric. Res. Serv., USDA, Washington, DC, USA, pp. 20pp.

Hall, M., Mertens, D.R., 2012. A ring test of in vitro neutral detergent fiber digestibility: Analytical variability and sample ranking. J. Dairy Sci. 95, 1992–2003.

Holden, L.A., 1999. Comparison of methods of in vitro dry matter digestibility for ten feeds. J. Dairy Sci. 82, 1791–1794.

Huhtanen, P., Nousiainen, J., Rinne, M., 2006. Recent developments in forage evaluation with special reference to practical applications. Agric. Food Sci. 15, 293–323.

Jones, D.I.H., Theodorou, M.K., 2000. Enzyme techniques for estimating digestibility. In: Givens, D.I., Owen, E., Omed, H.M., Axford, R.F.E. (Eds.), Forage Evaluation in Ruminant Nutrition. CABI Publishing, Wallingford, Oxfordshire, UK, pp. 155–173.

Julier, B., Lila, M., Furstoss, V., Travers, V., Huyghe, C., 1999. Measurement of cell-wall digestibility in lucerne using the filter bag technique. Anim. Feed Sci. Technol. 79, 239–245.

Lila, M., Barrière, Y., Traineau, R., 1986. Mise au point et étude d'un test enzymatique de la digestibilité de fourrages pauvres ou riches en amidon. Agronomie 6, 285–291.

Ludwin, J., 2007. Effectiveness of Assessment of Forage Digestibility by Enzymatic Method using the Filter Bags (in Polish). University of Agriculture in Krakow, PhD Thesis.

Mabjeesh, S.J., Cohen, M., Arieli, A., 2000. In vitro methods for measuring the dry matter digestibility of ruminant feedstuffs: Comparison of methods and inoculum source. J. Dairy Sci. 83, 2289–2294.

Menke, K.H., Steingass, H., 1988. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. Anim. Res. Dev. 28, 7–55.

Nousiainen, J., Rinne, M., Hellämäki, M., Huhtanen, P., 2003. Prediction of the digestibility of the primary growth and regrowth grass silages from chemical composition, pepsin-cellulase solubility and indigestible cell wall content. Anim. Feed Sci. Technol. 110, 61–74.

Robertson, J.B., Van Soest, P.J., 1981. The detergent system of analysis. In: James, W.P.T., Theander, O. (Eds.), The Analysis of Dietary Fibre in Food. Marcel Dekker, NY, pp. 123–158, Chapter 9.

SAS Institute, 2008. SAS/STAT® 9.2 User's Guide, The REG Procedure (Book Excerpt)., pp. 5517–5521.

Tilley, J.M., Terry, R.A., 1963. A two-stage technique for the in vitro digestion of forage crops. J. Br. Grassl. Soc. 18, 104–111.

Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fibre, neutral detergent fibre and non-starch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74, 3583–3597.

Vogel, K.P., Pedersen, J.F., Masterson, S.D., Toy, J.J., 1999. Evaluation of a filter bag system for NDF, ADF, and IVDMD forage analysis. Crop Sci. 39, 276–279.

Wilman, D.D., Adesogan, A., 2000. A comparison of filter bag methods with conventional tube methods of determining the in vitro digestibility of forages. Anim. Feed Sci. Technol. 84, 33–47.