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Research Article

Using *in vitro* methods to estimate metabolizable energy content of five forage legumes harvested under different defoliation systems

Einsatz von *in vitro* Methoden zur Schätzung der umsetzbaren Energie in fünf Futterleguminosen aus unterschiedlichen Nutzungssystemen

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Summary

Two *in vitro* methods were tested to establish their potential to predict the metabolizable energy (ME) content of forage legumes: the Tilley and Terry (TT) method and the pepsin-cellulase method (CM). Different samples of white clover (*Trifolium repens* L.), red clover (*Trifolium pratense* L.), kura clover (*Trifolium ambiguum* M. Bieb.), lucerne (*Medicago sativa* L.), and birdsfoot trefoil (*Lotus corniculatus* L.) were derived from field trials with several defoliation systems at two sites. The CM was more precise due to its repeatability within and between analysis runs, but eventually overestimated the ME contents of the samples, as it was shown for the standard samples with known *in vivo* digestibility. ME contents were found to be consistently higher based on CM, with a difference of up to 1.5 MJ ME/kg DM compared to TT. Although white clover was, in general, the species with the highest ME content, the influence of legume species over all cuts and defoliation systems was inconsistent. Such observations may influence the method of choice for ME estimation for large datasets.

Keywords: Forage legumes, metabolizable energy, NIRS, Tilley and Terry method, pepsin-cellulase method

Zusammenfassung

Zwei *in vitro* Methoden wurden dahingehend überprüft, das Potential des Gehaltes an umsetzbarer Energie (ME) von Futterleguminosen zu schätzen: die Tilley und Terry- (TT) und die Pepsin-Cellulase-Methode (CM). Unterschiedliche Proben aus Weißklee, Rotklee, Kuraklee, Luzerne und Hornklee wurden von Feldversuchen gewonnen, welche verschiedene Nutzungssysteme darstellen. Die CM-Methode war präziser durch die hohe Wiederholbarkeit zwischen und innerhalb der Analysenchargen, sowie für die Standardproben mit bekannter *in vivo* Verdaulichkeit. ME-Gehalte waren durchwegs höher wenn mit der CM-Methode ermittelt, mit einer Differenz von bis zu 1,5 MJ ME/kg TS im Vergleich zur TT-Methode. Auch wenn Weißklee die Spezies mit dem höchsten ME-Gehalt war, der Einfluss der Leguminosenart über alle Aufwüchse und Nutzungssysteme hinweg war nicht konsistent. Solche Beobachtungen beeinflussen die Wahl der Methode für die Ermittlung des ME-Gehaltes bei großen Datenmengen.

Schlagworte: Futterleguminosen, umsetzbare Energie, NIRS, Tilley und Terry-Methode, Pepsin-Cellulase-Methode

Abbreviations
OMD, organic matter digestibility; TT, Tilley and Terry method; CM, pepsin-cellulase method; ME, metabolizable energy; WC, white clover; RC, red clover; LC, lucerne; BT, birdsfoot trefoil; KC, kura clover; RG, rotational grazing; CM-OMD, OMD based on pepsin-cellulase method; TT-OMD, OMD based on Tilley and Terry method; ME_{CM}, ME estimated by pepsin-cellulase method; ME_{TT}, ME estimated by Tilley and Terry method; MAT, mean average temperature; AP, average precipitation.

1. Introduction

With the genetic advancement of new cultivars, improvement in the forage quality of legumes is expected to meet an improved animal performance. Both animal and forage plant-related factors are involved in the improvement. In animals, factors like breed, feed intake level and amount of concentrate in the diet will influence organic matter digestibility (OMD) of forages. In forage plants, agronomic traits like leaf:stem ratio, defoliation system and growth environment (site and year) may influence OMD. There is a need to do a systematic analysis of influencing factors to quantify the variation in feed quality prediction, especially the metabolizable energy (ME) content.
OMD seems to be the simplest way to compare the genetic progress in forage plants (Casler and Vogel, 1999). Currently, OMD is estimated using *in vitro* methods, which allows a large number of samples to be analyzed on a routine basis. OMD estimates from *in vitro* methods are validated using feeding trials to ensure predictability for a diversity of samples. Forages are subjected to higher variation in OMD; therefore, robust *in vitro* methods are required. Furthermore, OMD measurements are commonly included in prediction equations of ME content in forages in feed evaluation systems. The two methods for OMD determination are the Tilley and Terry method (TT) and the pepsin-cellulase method (CM); these methods are commonly used in different countries throughout Europe. Their advantages and limitations for application have been evaluated and thoroughly discussed for a range of forages in numerous articles and reviews (e.g., Ayres, 1991; Kitessa et al., 1999; Gosselink et al., 2004). In general, the main feature of the CM method is the independency of donor animals for rumen fluid, and its repeatability within and between runs. One constraint of the TT method is the variability in the quality of rumen fluid, as possible interactions between microbial species in the rumen of donor animals and the tested forages may occur. Inconsistencies among studies have been reported, as rumen fluid techniques have shown contrary findings compared to cellulase-based methods in the literature (Aufrère and Michalet-Doreau, 1988; De Boever et al., 1988; Givens et al., 1990; Steg et al., 1990). Comparable efficacy to predict OMD was observed as well (Gosselink et al., 2004).
Whereas numerous studies have shown a variation in the chemical composition of grasses depending on the growth season and development stage at harvest for prediction of ME content, the prediction of ME in forage legumes is poorly documented. Previous studies have shown a larger variation in the cell wall content of the perennial ryegrass growing in contrasting environments than forage legumes, and thus variable ME content (Gierus et al., 2007). Both TT and CM methods are endpoint measurements, i.e. both methods result in one replicable value that is used in the prediction equations. Therefore, it is hypothesized that both TT and CM are suitable for the prediction of ME of forage legumes growing in different defoliation systems, and both methods generate comparable ME values using OMD estimates with high precision for a range of forage legume species and harvest dates (the *in vitro* OMD estimates are validated beforehand with *in vivo* trials). Therefore, the objective of the present study was to estimate the ME content of five forage legumes, grown as binary legume–grass mixtures, and submitted to different defoliation systems, using *in vitro* methods.

2. Material and methods

2.1 Plant material

A total of 431 samples were derived from two field trials in Noer, Germany (16 m ASL; 8.7°C MAT; 774 mm AP) and Gumpenstein, Austria (710 m ASL; 6.8°C MAT; 1010 mm AP), and these were harvested for two years. The experimental designs at both sites were carried out as randomized block designs with three replicates each. Up to five legume species (white clover (cv. Klondyke), WC; red clover (cv. Pirat), RC; lucerne (cv. Ameristand), LC; birdsfoot trefoil (cv. Rocco), BT; kura clover (cv. Endura), KC) were grown in binary mixtures with perennial ryegrass (*Lolium perenne* L.). Three different defoliation systems were applied: (a) a 3-cut system (cut at every 50±5 days, first cutting after ear emergence of grass), established at both study sites; (b) a 5-cut system (cut at every 30±3 days, first

cut when the first node of grass is detectable); (c) rotational grazing (RG; nine animals were allowed to graze on plots of size 1500 m² for 3–5 days at intervals of 30±3 days). The management systems (b) and (c) were only established at the study site in Germany. Fresh plants were harvested keeping a cutting height of 5 cm and the growth was separated into grass and legume fractions. Samples were oven-dried at a temperature of 58°C and ground using a Cyclotech mill (Tecator, Haverhill, MA, USA) to a particle size of 1 mm.

All available legume samples were scanned twice using near infrared spectrometry (NIRS) Systems 5000 scanning monochromator (Perstrop Analytical Inc., Silver Spring, MD, USA) and software (ISI-version) for data collection and manipulation was supplied by Infrasoft International® (ISI, Port Matilda, PA, USA). Samples with H-values exceeding 3.0 were excluded from the calibration procedure. The calibration subsets that were selected (H-value 0.6) represented the whole sample spectrum, while the validation subsets were randomly selected after ranking the spectral data according to their H distance. Calibrations were developed by regressing laboratory determined values against the NIR spectral data (Shenk and Westerhaus, 1991). The minimum F statistics for terms included in the equation was 8.0. Subsets of samples were chosen for wet chemical analysis.

2.2 Nitrogen analyses

N was analysed by a rapid combustion (850°C), conversion of all N products to N_2, and subsequent measurement by thermoconductivity cell (elementar-analysator Vario MAX CN, Fa. Elementar Analysensysteme, Hanau, Germany). The results were expressed as crude protein, i.e. N × 6.25.

2.3 Pepsin-cellulase method

The pepsin-cellulase method (CM) was carried out according to De Boever et al. (1988) in Germany, following the guidelines of VDLUFA Standard Methodology (VDLUFA, 1993). Briefly, the method involved a preliminary incubation for 24 h with pepsin/HCl at 40°C, followed by heating for 45 min at 80°C and a second incubation with a commercial cellulase Onozuka R-10 from *Trichoderma viride* (Merck, Darmstadt, Germany).

The ME content by the CM method (ME_{CM}) was computed by applying the estimating equation for legumes derived by Weissbach et al. (1996) on the values obtained with the *in vitro* method:

(1) ME_{CM} (MJ/kg DM) = 13.98 – 0.0147 × CA – 0.0137 × IOM + 0.00234 × CP,

where CA is crude ash content (g/kg DM), IOM is enzymatically insoluble organic matter (g/kg DM) and CP is crude protein content (g/kg DM).

Four standard samples with known *in vivo* digestibility values were included for *in vitro* analyses. Single standard samples were randomly included per run in quadruplicate. Results of the standard samples were calculated as cellulase organic matter digestibility (CM-OMD; % DM), based on the calculated loss upon ashing (L; g), initial weight of the sample (W; g), dry matter content (DM; %) and ash content (CA; %) (VDLUFA 1993).

(2) CM-OMD (% DM) = 100 – (L × 1 000 000) / (W × DM × (100 – CA))

2.4 Two-stage method by Tilley and Terry

The two-stage method by Tilley and Terry (1963) (TT) was carried out with modifications in Austria. Rumen fluid was obtained prior to the morning feeding from two rumen-fistulated steers fed with a diet of seasonal green forage from mixed swards and supplemented with concentrates. The buffer solution was dispensed according to McDougall (1948). Prior to incubation, rumen fluid and buffer solution were mixed in the proportion 1:4 (v/v). The pepsin solution was prepared by dissolving 20 g of 1:10,000 pepsin (Sigma-Aldrich, Germany) in 1000 ml distilled water. Where required, steps were carried out under anaerobic conditions, flushing buffers and solutions with gaseous CO_2.

Dried forage samples (0.5 g) were weighed in triplicate into 100 ml Erlenmeyer flasks and 50 ml of the rumen liquor-buffer solution were added. Remaining air was then expelled with CO_2 and flasks sealed with perforated Parafilm, followed by incubation of samples and blanks at 38.5°C for 48 h in the dark. At the end of the first incubation period, pH value was adjusted to 1.5 units by using 2.2N HCl, 5 ml of pepsin solution was added and then the flasks were incubated again for 48 h at 38.5°C. At the end of the second incubation period, samples were filtered (Macherey Nagel MN 640w, Germany), dried at 104°C for 4 h and weighed before ashing at 450°C.

The TT-OMD was calculated as difference between the OM of the sample before incubation and the residual OM. Residues after incubation measured in blanks were deducted. Outliers (if the deviation of a replicate exceeded 3 % of the mean) were excluded from further calculations.

The same four standard samples with known *in vivo* digestibility values, similar to the CM method, were included for the *in vitro* TT analyses, and all standard samples were analyzed in six replicates within each run.

The TT-OMD values of the standard samples were compared within each run to their corresponding *in vivo* values by linear regression. A correction equation for *in vitro* values of the samples was generated in terms of variability due to rumen fluid quality between the runs, and to express the results of the samples as estimated *in vivo* OMD. ME content for the samples in the present experiment was estimated by regression analysis of the analyzed TT-OMD (g/kg DM) with benchmark values obtained from the Deutsche Landwirtschaftsgesellschaft (DLG) tables (DLG, 1997). The benchmark values published in the tables originate from *in vivo* trials with lactating cows. Regression analyses were performed separately for the first cut, and then for subsequent regrowths on data of grassland with high proportion of legumes, giving the following equations:

(3) 1st cut: ME_{TT} (MJ/kg DM) = 0.0174 × TT-OMD – 1.2677; R^2 = 0.99; *SEM* = 0.07

(4) regrowths: ME_{TT} (MJ/kg DM) = 0.0172 × TT-OMD – 1.0306; R^2 = 0.99; *SEM* = 0.08

2.5 Statistical analysis

A mixed model analysis was calculated for energy content data (ME) of each defoliation system and for the 3-cut system of both sites using PROC MIXED by considering cut, species and method as fixed factors (SAS Institute Inc., 2004). Years and site (data was available for the 3-cut system in both sites) were included in the data set, not as classificatory factors in the statistical model, but considered as replicates. Cuts (or grazing cycles) were treated as a repeated measurement assuming a symmetric covariance structure. In case of significant interactions ($P < 0.05$), linear contrasts were calculated using the SLICE procedure of SAS. Comparison of least squares means were performed by t-test. To avoid random significances, probabilities were adjusted by the Bonferroni–Holm procedure at $P < 0.05$ (Holm, 1979).

The relationship between the NIRS-estimated ME contents based on the two different *in vitro* methods was determined by linear regression analysis for different factor combinations (forage legume, defoliation system and harvest season). Parameters of the determined regression equations (slope, intercept) were tested to evaluate whether they differed from unity or zero, respectively.

3. Results

Table 1 shows the descriptive statistics of the NIRS calibration results for all samples included in the evaluation, giving the range of estimated values for ME contents of the respective methods (ME_{CM}; ME_{TT}). Additionally, the variation coefficient referred to as the standard deviation (CV_{SD} = SEC × 100/SD) was calculated in order to assess the suitability of the respective method for reliable NIRS calibration, as suggested by Murray (1986). The NIRS

Table 1. NIRS calibration statistics of the determined parameters IOM (g/kg DM), ME_{CM} (MJ/kg DM), TT-OMD (%), and ME_{TT} (MJ/kg DM) of the legume samples (n = 431)

Tabelle 1. NIRS Kalibrationsstatistik für folgende Parameter: IOM (g/kg TM), ME_{CM} (MJ/kg TM), TT-DOM (%) und ME_{TT} (MJ/kg TM)

Parameter	n (1)	Range (2)		Mean (3)	SD (4)	SEC (5)	R^2 (6)	CV_{SD} (7)
IOM (8)	77	90.60	353.73	204.42	72.18	11.52	0.98	15.96
ME_{CM}	76	8.18	11.90	10.11	0.93	0.15	0.97	16.27
TT-OMD	77	42.42	79.83	63.51	7.82	3.24	0.83	41.43
ME_{TT}	73	6.20	11.03	8.61	1.05	0.59	0.69	55.86

(1) Number of samples included in the calibration

(2) Minimum and maximum of the parameter values

(3) Mean of the parameter values

(4) Standard deviation of the laboratory-determined values

(5) Standard error of calibration

(6) Coefficient of determination; relationship between NIRS- and laboratory-determined values

(7) Variation coefficient referred to the SD of the reference method ($CV_{SD} = SEC \times 100/SD$)

(8) IOM, enzymatically insoluble organic matter; ME_{CM}, ME content estimated with pepsin-cellulase method; TT-OMD, Tilley and Terry method estimated organic matter digestibility; ME_{TT}, ME content estimated using Tilley and Terry method data

calibration models obtained correlations with all nutrient and energy variables analyzed with $R^2 > 0.80$, with the exception of ME_{TT} with a considerably lower R^2 of 0.69, compared to ME_{CM} ($R^2 = 0.97$). The calibration model for ME_{TT} is thus classified as poor. Estimation of the *in vitro* parameters (as used in the ME estimations) resulted in a more precise prediction of IOM ($R^2 = 0.98$), whereas the TT-OMD had a lower R^2 (0.83), with a CV_{SD} value of 41% (Table 1).

Table 2 shows the *in vivo* values of the four standard samples as well as the descriptive statistics of their *in vitro* analyses, calculated as differences of the *in vitro* analyzed OMD (CM-OMD and TT-OMD) to their *in vivo* digestibility. Differences of TT-OMD for the standard samples included a wide range for the OMD estimates, with a poorer accuracy indicated by remarkably large min–max differences. Means of TT-OMD ranged from –2.4 to –3.5% points with a comparably higher SD, indicating a general underestimation of the OMD estimated by TT compared to the CM method. Consequently, a less precise ME estimation was observed using the TT method.

Results of the statistical evaluation of the 2-way interactions are given in Table 3 for the 3-cut system at both study sites, and in Table 4 for the study site in Germany, separated for each defoliation system. The 2-way interactions species × cut, method × cut and method × species were significant (Table 3 and 4). In general, lower ME values were estimated based on TT compared to the CM method, with LSMeans being different ($P < 0.05$) between methods within species, and between methods within cut.

For the dataset including both study sites (Table 3), higher ME values as means over cuts were consistently estimated for white clover, followed by RC in both sites. As means over species, forage of the first cut showed highest ME values, and lowest values were seen in the second cut. However, the range of ME for the species was only slightly larger within the ME_{TT} dataset (1.5 MJ) than within ME_{CM} (1.1 MJ). Averaged over methods, ME values of most species did not differ between the second and third cut, but ME of BT was lower in the third cut (8.4 MJ). Such difference among species in the third cut was not detected by the methods individually.

ME_{CM} contents of forage legumes were always higher ($P < 0.05$) than ME_{TT} for the samples collected from the study site in Germany (Table 4). KC was equal to WC when estimated by ME_{CM} in the 3-cut system, but was different from WC in both cut systems using ME_{TT}. Among all systems, WC showed the highest ME content, for both CM and TT methods. Estimated as ME_{CM}, energy contents as means over cuts declined from first to the third cut, whereas ME_{TT} was equal in the second and third cut.

Data of the 5-cut system showed consistency in the ranking of species within the methods as means over cuts, with all species differing from each other, giving the highest ME for WC > KC > RC > LC > BT. This was similar for the RG system as well. Comparing the ME_{CM} and ME_{TT} for each cut and species in the 5-cut system and the RG system, the ME values showed lower differences within the first cutting dates, whereas larger deviations could be observed in the fourth and fifth cut. In the 5-cut system, ME_{CM} of RC, LC and BT were high in the forage of the fourth and fifth cut, whereas ME_{TT} was similar or lower.

Figure 1 shows the relationship between ME of all samples included in the evaluation ($n = 149$). The generally smaller

Table 2. Differences between the *in vitro* analyzed OMD (CM-OMD: pepsin-cellulase method; TT-OMD: Tilley and Terry method) of four standard samples and their *in vivo* digestibility; TT-OMD data prior to routine correction with *in vivo* OMD

Tabelle 2. Differenzen der *in vitro* untersuchten OMD (CM-OMD: Pepsin-Cellulase-Methode; TT-OMD: Tilley und Terry-Methode) für vier Standardproben zu *in vivo* Verdaulichkeit; TT-OMD Daten vor der Routinekorrektur durch die *in vivo* OMD

Standard samples			*in vivo* OMD±SD	CM-OMD (%)					TT-OMD (%)				
No.	Cuts/year	Cut	(%)	*n* (1)	Range (2) Min	Range (2) Max	Mean (3)	SD (4)	*n* (1)	Range (2) Min	Range (2) Max	Mean (3)	SD (4)
198	1	1	41.9±3.92	5	0.72	3.30	2.06	1.13	24	–15.44	1.89	–2.44	3.99
232	3	1	68.4±2.13	5	–3.91	–2.88	–3.63	0.43	24	–10.82	1.09	–3.45	3.16
246	4	2	75.2±2.26	4	2.92	4.34	3.78	0.63	24	–7.74	0.93	–3.50	3.07
298	1	1	46.7±4.15	5	2.65	3.65	2.98	0.40	23	–15.56	1.22	–3.20	4.68

(1) Number of runs in which standard samples was included

(2) Minimum and maximum differences to the *in vivo* value

(3) Mean of the differences to the *in vivo* value

(4) Standard deviation of the differences of the laboratory-determined values to the *in vivo* values

Table 3. ME contents of several forage legumes from the 3-cut system at three cutting dates over study sites and years, based on two different *in vitro* methods (n = 286), expressed as MJ ME/kg DM
Tabelle 3. ME Gehalt verschiedener Futterleguminosen (3-Schnittsystem) als Mittelwert über Standorte und Jahre, mittels zwei *in vitro* Methoden (n = 286), als MJ ME/kg TS

	Pepsin-cellulase method (ME_{CM})				Tilley and Terry method (ME_{TT})				Means over methods		
	Cut 1	Cut 2	Cut 3	*Mean*	Cut 1	Cut 2	Cut 3	*Mean*	Cut 1	Cut 2	Cut 3
White clover	11.0	10.1	10.6	10.6 ^{v,V}	10.1	8.8	9.4	9.4 ^{w,V}	10.5 ^{a,A}	9.4 ^{a,C}	10.0 ^{a,B}
Red clover	10.3	9.7	10.0	10.0 ^{v,W}	9.2	8.2	8.8	8.7 ^{w,W}	9.8 ^{b,A}	8.9 ^{b,C}	9.4 ^{b,B}
Lucerne	9.9	9.5	9.5	9.6 ^{v,X}	8.3	7.6	7.8	7.9 ^{w,X}	9.1 ^{c,A}	8.5 ^{c,B}	8.6 ^{c,B}
Birdsfoot trefoil	9.9	9.3	9.4	9.5 ^{v,X}	8.5	7.6	7.5	7.9 ^{w,X}	9.2 ^{c,A}	8.5 ^{c,B}	8.4 ^{d,B}
Mean	10.3 ^{g,G}	9.6 ^{g,I}	9.9 ^{g,H}	9.9	9.0 ^{h,G}	8.1 ^{h,I}	8.4 ^{h,H}	8.5			

^{a,b,c,d} LSMeans differ between species within cutting date at $P < 0.05$; $SE = 0.07$
^{A,B,C} LSMeans differ between cutting dates within species at $P < 0.05$; $SE = 0.07$
^{g,h} LSMeans differ between methods within cutting date at $P < 0.05$; $SE = 0.05$
^{G,H,I} LSMeans differ between cutting date within method at $P < 0.05$; $SE = 0.05$
^{v,w} LSMeans differ between methods within species at $P < 0.05$; $SE = 0.05$
^{V,W,X,Y} LSMeans differ between species within method at $P < 0.05$; $SE = 0.05$

ME_{TT} values observed were reflected in the regression equation, the intercept (–1.35) differing from zero ($P < 0.05$), whereas the slope did not differ from unity. Large variation around the regression line resulted in a relatively poor correlation coefficient ($R^2 = 0.63$). Separation by defoliation systems (Figure 2) did not improve the results compared to the whole dataset (in Figure 1). Only the 3-cut system (Figure A) showed reasonable prediction.

4. Discussion

The main focus of the present study was on the measurement of the repeatability of ME estimation of two *in vitro* methods combining the following factors: forage legume species, cut; or forage legume species, and defoliation system. Since both methods have been validated throughout the literature for their suitability to estimate *in vivo* measurements of OMD, less effort was required for validation of the outcome with *in vivo* measurements of OMD in this study.

4.1 Within and between methods variation

As shown by the mixed model and regression analyses of all data, the determination of ME of forage legumes clearly differed depending on the *in vitro* method used, with systematic higher values predicted using the CM method. The general limitations given within the methods and the respective equations are possible reasons for this study. Whereas OMD values obtained with TT are dependent on the variation obtained for the standard samples measured simultaneously in each run, the CM method does not need simultaneous measurement of standard samples, as the equation is based on several *in vivo* digestibility trials. Although a low number of samples originated from *in vivo* trials, the weak repeatability in TT could be due to methodological aspects of the *in vivo* digestibility method, or due to the variations accounted for, for example, the harvest time or botanical composition of the standard samples.

For the CM method, a regression equation was developed for ME estimation separately for legumes by Weissbach et al. (1996). This equation is based on 20 *in vivo* digestibility trials, including LC and RC, preserved as silage, hay and oven-dried forage, in all covering a range of 29 to 77% OMD. As found in other studies, the *in vitro* CM-OMD values generally agreed with *in vivo* values, regardless of the feed type analyzed (De Boever et al., 1988). The larger variation of values obtained from the TT procedure was reflected in the larger variation of results of the standard sample analysis (Table 2). Although the difference to the *in vivo* OMD were lower, the ME estimates based on TT gave systematically lower ME values when considering cuts, legume species and management types compared to the CM method in the present study.

Table 4. ME contents of forage legumes from different defoliation systems (3-cut system, 3C; 5-cut system, 5C; rotational grazing, RG) from the site in Germany over years, estimated by NIRS based on two different *in vitro* methods, expressed as MJ ME/kg DM

Tabelle 4. ME Gehalte von Futterleguminosen verschiedener Nutzungssystems (3-Schnitt-, 3C; 5-Schnittsystem, 5C; Umtriebsweide, RG) aus dem Standort Deutschland als mittel über Jahre, geschätzt mittels NIRS aus zwei unterschiedlichen *in vitro* Methoden, als MJ ME/kg TS

	Pepsin-cellulase method (ME_{CM})						Tilley and Terry method (ME_{TT})						Means over methods				
	Cut 1	Cut 2	Cut 3	Cut 4	Cut 5	*Mean*	Cut 1	Cut 2	Cut 3	Cut 4	Cut 5	*Mean*	Cut 1	Cut 2	Cut 3	Cut 4	Cut 5
3-cut system (n = 176)																	
WC	11.0	10.0	10.2			10.4 ^{v,V}	10.6	8.8	9.3			9.6 ^{w,V}	10.8 ^{a,A}	9.4 ^{a,C}	9.8 ^{a,B}		
RC	10.4	9.7	9.4			9.8 ^{v,W}	9.6	8.3	8.6			8.8 ^{w,X}	10.0 ^{c,A}	9.0 ^{b,B}	9.0 ^{b,B}		
KC	10.8	10.3	10.3			10.5 ^{v,V}	9.9	9.0	8.9			9.2 ^{w,W}	10.3 ^{b,A}	9.6 ^{a,B}	9.6 ^{a,B}		
LC	9.8	9.5	9.0			9.4 ^{v,X}	8.4	7.7	7.4			7.8 ^{w,Y}	9.1 ^{e,A}	8.6 ^{c,B}	8.2 ^{c,C}		
BT	10.0	9.7	8.9			9.6 ^{v,X}	9.1	7.9	7.0			8.0 ^{w,Y}	9.6 ^{d,A}	8.8 ^{bc,B}	8.0 ^{c,C}		
Mean	10.4 ^{g,G}	9.8 ^{g,H}	9.6 ^{g,I}			9.9	9.5 ^{h,G}	8.3 ^{h,H}	8.2 ^{h,H}			8.7					
5-cut system (n = 276)																	
WC	11.8	11.1	9.8	11.1	11.2	11.0 ^{v,V}	10.1	9.9	8.6	9.7	9.5	9.5 ^{w,V}	10.9 ^{a,A}	10.5 ^{a,B}	9.2 ^{b,C}	10.4 ^{a,B}	10.4 ^{a,B}
RC	11.2	10.8	9.9	10.4	10.7	10.6 ^{v,X}	9.6	9.5	8.4	8.8	8.6	9.0 ^{w,X}	10.4 ^{b,A}	10.1 ^{b,B}	9.1 ^{b,D}	9.6 ^{c,C}	9.6 ^{c,C}
KC	11.4	10.9	10.5	10.9	10.8	10.9 ^{v,W}	9.6	9.5	9.0	9.4	9.2	9.3 ^{w,W}	10.5 ^{b,A}	10.2 ^{b,B}	9.7 ^{a,C}	10.1 ^{b,B}	10.0 ^{b,B}
LC	10.8	10.4	10.0	10.1	10.4	10.4 ^{v,Y}	8.8	8.6	8.0	8.2	8.2	8.4 ^{w,Y}	9.8 ^{c,A}	9.5 ^{c,B}	9.0 ^{b,D}	9.2 ^{d,CD}	9.3 ^{d,BC}
BT	10.9	9.9	9.8	10.1	10.4	10.2 ^{v,Z}	8.5	7.9	7.6	7.9	8.0	8.0 ^{w,Z}	9.7 ^{c,A}	8.9 ^{d,C}	8.7 ^{c,D}	9.0 ^{e,C}	9.2 ^{d,B}
Mean	11.2 ^{g,G}	10.6 ^{g,HI}	10.0 ^{g,J}	10.5 ^{g,I}	10.7 ^{g,H}	10.6	9.3 ^{h,G}	9.1 ^{h,H}	8.3 ^{h,J}	8.8 ^{h,I}	8.7 ^{h,I}	8.8					
RG (n = 238)																	
WC	11.5	10.9	10.6	11.2	11.5	11.1 ^{v,V}	10.5	9.7	9.5	9.7	9.4	9.8 ^{w,V}	11.0 ^{a,A}	10.3 ^{a,B}	10.0 ^{a,C}	10.5 ^{a,B}	10.4 ^{a,B}
RC	11.1	11.1	10.1	10.5	10.6	10.7 ^{v,W}	9.6	9.6	8.8	8.7	8.7	9.1 ^{w,W}	10.4 ^{b,A}	10.4 ^{a,A}	9.5 ^{b,B}	9.6 ^{b,B}	9.6 ^{b,B}
LC	10.6	10.5	10.4	10.2	10.4	10.4 ^{v,X}	8.7	8.8	8.7	8.2	8.1	8.5 ^{w,X}	9.6 ^{c,A}	9.7 ^{b,A}	9.6 ^{b,A}	9.2 ^{c,B}	9.2 ^{c,B}
BT	9.9	10.2	9.7	10.1	10.4	10.1 ^{v,Y}	8.2	8.3	7.8	7.7	8.1	8.0 ^{w,Y}	9.1 ^{d,AB}	9.3 ^{c,A}	8.7 ^{c,C}	8.9 ^{d,BC}	9.3 ^{c,A}
Mean	10.8 ^{g,G}	10.7 ^{g,G}	10.2 ^{g,I}	10.5 ^{g,H}	10.7 ^{g,G}	10.6	9.2 ^{h,G}	9.1 ^{h,G}	8.7 ^{h,H}	8.6 ^{h,H}	8.6 ^{h,H}	8.8					

^{a,b,c,d,e} LSMeans differ between species within cutting date at $P < 0.05$; $SE_{3C} = 0.12$; $SE_{5C} = 0.08$; $SE_{RG} = 0.08$

^{A,B,C,D} LSMeans differ between cutting dates within species at $P < 0.05$; $SE_{3C} = 0.12$; $SE_{5C} = 0.08$; $SE_{RG} = 0.08$

^{g,h} LSMeans differ between methods within cutting date at $P < 0.05$; $SE_{3C} = 0.07$; $SE_{5C} = 0.04$; $SE_{RG} = 0.05$

^{G,H,I,J} LSMeans differ between cutting date within method at $P < 0.05$; $SE_{3C} = 0.07$; $SE_{5C} = 0.04$; $SE_{RG} = 0.05$

^{v,w} LSMeans differ between methods within species at $P < 0.05$; $SE_{3C} = 0.09$; $SE_{5C} = 0.04$; $SE_{RG} = 0.04$

^{V,W,X,Y,Z} LSMeans differ between species within method at $P < 0.05$; $SE_{3C} = 0.09$; $SE_{5C} = 0.04$; $SE_{RG} = 0.04$

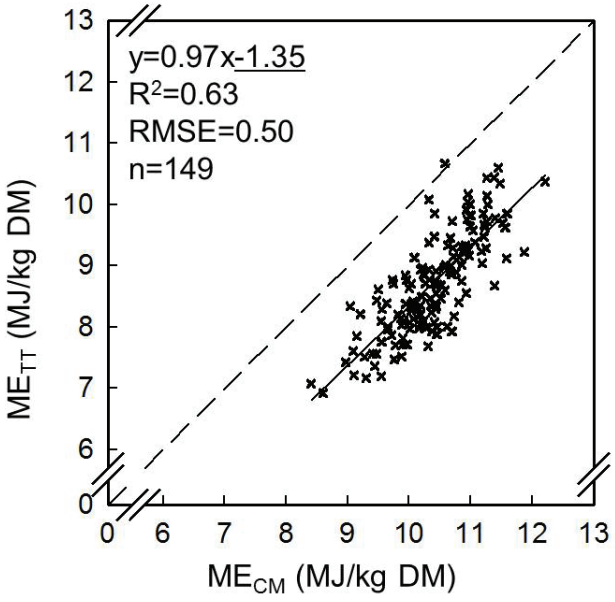

Figure 1. Relationship between ME of several forage legumes estimated by NIRS based on pepsin-cellulase method (ME_{CM}) and Tilley and Terry method (ME_{TT}), respectively. Figures include all data of both study sites and years (if available) as means over field replicates. Bisector shown as dashed line. Underlined regression parameters differ from unity (slope) respective zero (intercept).
Abbildung 1. Zusammenhang zwischen mit NIRS geschätzte ME-Gehalt verschiedener Futterleguminosen, auf Basis von der Pepsin-cellulase--Methode (ME_{CM}) bzw. Tilley und Terry-Methode (ME_{TT}). Die Abbildung zeigt die Daten aus beiden Standorte und Jahre (soweit verfügbar) als Mittelwerte über Feldwiederholungen. Die Winkelhalbierende wird als gestrichelte Linie dargestellt. Unterstrichene Regressionsparameter unterscheiden von Eins (Steigung) bzw. von Null (Achsenabschnitt).

4.2 Influence of the defoliation system

The defoliation frequency caused deviations of the intercepts, as the 5-cut system and RG were larger than zero (Figure 2). Compared to the 3-cut system, the plants in the 5-cut system and RG were harvested at an earlier development stage and thus showed high ME values (Table 4), as confirmed in previous experiments including grazing management (Kleen et al., 2011). The differences were consistent between sites and years.
Enzyme-based predictions of *in vivo* digestibility and ME content can vary with forage species and harvest season (Barber et al., 1990; Givens et al., 1995). Differences between harvest seasons were observed in *in vivo* digestibility equations for grasses using either enzyme or rumen inoculum based methods (Jones and Theodorou, 2000). Season influence on ME content is quite often observed, but less often for forage legumes. In the present study, the lowest content of ME was observed for the CM method in the third cut for the 5-cut system and RG (Table 4). In the autumn harvest, higher ME with the CM method was estimated to be comparable to the summer harvest, whereas with the TT method only a slight increase could be observed. For animal nutrition, the first harvest is quantitatively and qualitatively more important. From the second cut onwards, similar values for the ME content among cuts are mostly assumed, irrespective of management. However, the results suggested that the TT method may also underestimate the ME content in cuts late in the season (e.g., in autumn), especially at a higher defoliation frequency (Table 4), and undergrazing (Figure 2). Although a higher ME content in autumn harvests was observed, the way to use the grassland in this season is disputable, as the higher ME content is masked by low DM yield, higher forage legume proportion and poor protein quality (Kleen et al., 2011; Krawutschke et al., 2013), which may result in higher N losses in the production system, especially under grazing.

4.3 Influence of legume species

Forage legumes may have leaves with higher digestibility than stems, compared to grasses. In this case, management systems resulting in higher leaf:stem ratio may substantially improve OMD in legumes with erect, crown-forming growing habit (Annicchiarico, 2007). For legumes like LC, RC or BT, higher leaf:stem proportions are observed for higher cutting frequency (Gierus et al., 2012). Thus, these legumes have a stronger influence on higher digestibility and ME content due to an altered leaf:stem ratio with low maturity at harvest in the 5-cut system in the present study. Ranking legumes based on the leaf:stem proportion as used in the present study (Table 3 and 4) confirms the observations by others (Nordkvist and Åman, 1986). LC samples confirmed having a higher ME content in the 5-cut system and RG system in comparison to the 3-cut system. Achieving higher leaf:stem proportion would be a management option to improve forage quality for certain forage legume species with erect, crown-forming growth habit. However, the prediction of ME of several legumes submitted to different management systems shows that the largest ME values were observed for white clover, while comparing the 3-cut system at both sites (Table 3) or among defoliation systems in Germany (Table 4). Both CM and TT methods were able to measure the higher ME content for white clover consistently in Austria and Germany.

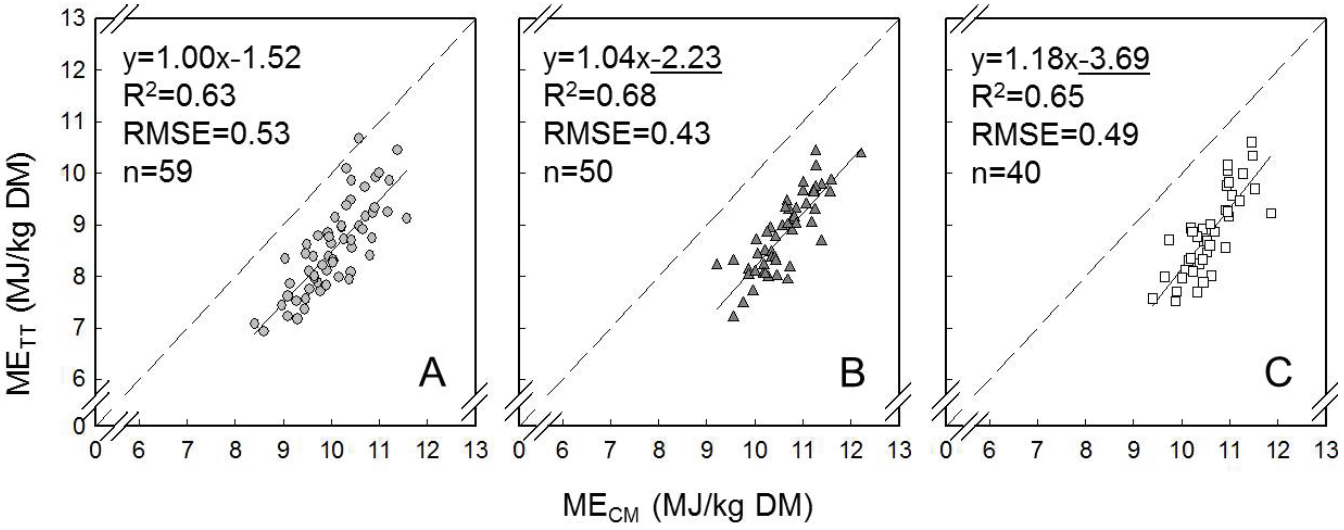

Figure 2. Relationship between ME of several forage legumes estimated by NIRS based on pepsin-cellulase method (ME_{CM}) and Tilley and Terry method (ME_{TT}). Figures include data from both study sites and years as means over field replicates, grouped by defoliation systems: A, 3-cut system; B, 5-cut system; C, rotational grazing. Bisector shown as dashed line. Underlined regression parameters differ from unity (slope) respective zero (intercept).

Abbildung 2. Zusammenhang zwischen mit NIRS geschätzte ME-Gehalt verschiedener Futterleguminosen, auf Basis von der Pepsin-Cellulase--Methode (ME_{CM}) bzw. Tilley und Terry-Methode (ME_{TT}). Die Abbildung zeigt die Daten beider Standorte und Jahre als Mittelwerte über Feldwiederholungen, sortiert nach den Nutzungssysteme: A, 3-Schnittsystem; B, 5-Schnittsysem; C, Umtriebsweide. Die Winkelhalbierende wird als gestrichelte Linie dargestellt. Unterstrichene Regressionsparameter unterscheiden von Eins (Steigung) bzw. von Null (Achsenabschnitt).

The comparison of ME_{TT} and ME_{CM} for individual data within legume species reveals that the higher ME values were equally well predicted compared to the lower ME contents for different legume species. Although higher in ME content, white clover did not show better prediction using either TT or CM in comparison to other species. Using legume forage samples with known *in vivo* OMD revealed that the CM method may overestimate the ME contents of the samples, whereas the TT method may underestimate the values. However, white clover (at both sites), together with KC (in Germany only) were the species with the highest ME content, independent of season (cut number), defoliation system, or site, which was confirmed using both methods.

4.4 Secondary compounds in forage legumes

Compared to other forage legumes, the enzyme polyphenol oxidase is very active in RC (Eickler et al., 2011). Using substrates like caffeic acid, phaselic acid and clovamide present in the plant, the enzyme catalyses the reaction and produces quinones and in this way may cause a complexation of proteins (Jones et al., 1995). The complexation is comparable to that observed for condensed tannins, which are present in BT, and the extent of their presence is dependent on their concentration in forage and diet. One may suggest that condensed tannins or quinones cause a stronger impact on the rumen microbes when the TT method is the method of choice, resembling the determination of *in vivo* OMD with non-fitted ruminal microflora of animals to tannins. The effect may be ignored in the CM method due to an unsuitable pH value in the pepsin step, and seems to be comparable to the OMD estimation in animals already adapted to secondary compounds. However, these differences are not always observed in the literature. Reviewed by Aufrère and Guérin (1996), prediction of OMD by enzymatic methods is, in general, accurate. For forages containing tannins, enzymatic methods overestimate OMD compared to *in vivo* digestibility, because of lower digestion *in vitro* due to the presence of tannins. Similar effects might occur in RC containing polyphenol oxidase activity, as the produced quinones may act in some cases similarly as condensed tannins.

The effect of polyphenols, either condensed tannins in BT or quinones in RC, on an annual basis was not apparent in the present study for the ME estimate. The ME estimates among legume species from TT and CM methods were lowest for BT and comparable for LC (Table 3

and 4), whereas the ME estimates of RC were only lower than white clover. The secondary plant components, quinones and condensed tannins, may have varied in their contents during the growing season, which was supported by the observed species × cut interactions over methods in the present study, and was also observed in other studies (Eickler et al., 2011). However, the variation was small but enough to show the influence of species (RC and BT) on a lower ME estimation as average over methods. This may be related to the content of condensed tannins or quinones formed within these species.

4.5 Feeding trials

While *in vitro* methods are suitable for the prediction of ME content of forages, *in vivo* are used to validate the predictions. Variation may arise due to limitations of *in vitro* analysis to include deviations of OMD due to feed intake level, diet selection of species and plant organs or animal species. In addition, *in vitro* methods may accumulate fermentation end products and may not consider the effects of passage rate, neither solid nor liquid phase (Dijkstra et al., 2005). However, feeding trials performed to quantify the level of inclusion of forage legumes on animal performance response showed controversial results (Steinshamn, 2010). For the present study, the large number of samples to test variations due to site, harvest date and defoliation system made the *in vivo* validation considerably more difficult, especially under grazing conditions. However, the present results supported the assumption that *in vitro* estimation methods reflect the ME content in forage legumes in the following order of decreasing relevance: legume species, defoliation system and harvest date.

5. Conclusions

For forage legumes, the first cut of the year is the most important one in terms of ME content. Especially for white clover and KC, as they have the highest ME content, independent of management system. Achieving a higher leaf:stem proportion, for example by higher defoliation frequency, would be a management option to improve forage quality for the investigated forage legume species.

The estimation of ME contents of forage legumes based on the CM method is far more robust due to the higher precision and correlation to *in vivo* values for large datasets. Cuts late in the season, especially at a higher defoliation frequency, caused the TT method to underestimate the ME content. Besides methodological aspects inherent to each approach, both CM and TT are suitable to estimate the ME content of forage legumes.

In vitro estimation methods reflect the ME content in forage legumes in the following order of decreasing relevance: legume species, defoliation system and harvest date.

In vivo trials are necessary to validate the variations detected by *in vitro* methods due to cut, defoliation method and site. However, the comparison with *in vivo* methods between defoliation systems may be difficult, as the OMD in grazing trials is estimated with markers.

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