A comparison of *in situ* and *in vitro* methods to estimate *in vivo* fermentable organic matter of forages in ruminants

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Abstract

Several known *in situ* and *in vitro* methods were compared for their reliability for determining – directly or indirectly – *in vivo* fermentable organic matter (*in vivo* FOM) of forages in ruminants. Twelve forage types were used: fresh and conserved forms of lucerne, red clover, orchard grass and perennial ryegrass. Organic matter truly digested in the rumen – which in our study was regarded as equivalent to *in vivo* FOM – was determined in six cannulated sheep, using the flow markers ^{51}Cr-EDTA and ^{103}Ru-Phenanthrolin. *In vivo* FOM was estimated directly from results of the *in situ* nylon bag technique using three cows, and from the results of three *in vitro* methods, and indirectly by calculating *in vivo* FOM using equations from the Dutch and French protein evaluation systems. The *in vitro* methods were an enzymatic technique using pepsin and cellulase, the method of Tilley & Terry and the gas production technique. *In vivo* FOM was best correlated (R^2 = 0.74; n = 12) with gas production after 20 hours of incubation. The correlation improved when fresh and conserved forages were considered separately (R^2 = 0.90; n = 12). Indirectly, *in vivo* FOM was well estimated from the results of the *in situ*, the gas production and the Tilley & Terry methods (R^2 = 0.76–0.80; n = 12). The accuracy of the direct and indirect *in vivo* FOM estimates was similar. However, the direct *in vivo* FOM estimate was a regression and the indirect estimate was a validation. In conclusion, *in vivo* FOM was best estimated indirectly using the equation from the Dutch protein evaluation system, whereas the estimate was more accurate with the *in situ* and the gas production techniques than when the other *in vitro* methods were used.

Additional keywords: nylon bag, gas production, Tilley & Terry, pepsin, cellulase, fermentable organic matter

Introduction

In vivo fermentable organic matter (*in vivo* FOM) of forages is a good measure of energy production in the rumen, an important factor for determining the potential synthesis of microbial protein in the rumen. Measurements of *in vivo* FOM with fistulated animals are expensive and laborious and negatively affect animal welfare. In French and Dutch protein evaluation systems (Vérité *et al.*, 1987; Tamminga *et al.*, 1994) fermentable organic matter (FOM) is calculated from organic matter total tract digestibility (OMD). In the past 40 years great efforts have been made to develop alternative methods to measure fermentable or degradable OM (alternative DOM) in order to estimate *in vivo* FOM.

The most frequently used methods to measure alternative DOM are the *in situ* (nylon bag) and the gas production technique, but also methods such as the pepsin-cellulase (Aufrère & Demarquilly, 1989) and the *in vitro* method of Tilley & Terry (1963) can be used. These four methods yield results that are well correlated with OMD measured in animals (*in vivo* OMD) (Tilley & Terry, 1963; Aufère & Michalet-Doreau, 1988; Menke & Steingass, 1988; Fonseca *et al.*, 1998). Although methods have been compared (Givens *et al.*, 1989; Blümmel & Ørskov, 1993; Cone *et al.*, 1999; Chenost *et al.*, 2001), the comparisons were usually in pairs or different procedures were used for the same technique. Evaluations of *in situ* and *in vitro* techniques as estimators of *in vivo* FOM are scarce. The *in situ* method has been related to *in vivo* FOM in a study using a variety of feedstuffs (Arieli *et al.*, 1998). Rymer & Givens (2002) compared patterns of rumen fermentation measured with the *in situ* and the gas production technique.

To correlate alternative DOM with *in vivo* FOM is more difficult than to correlate it with *in vivo* OMD. Firstly, much more *in vivo* OMD data are available because it is easier to measure than *in vivo* FOM. Secondly, FOM depends on rumen dynamic processes, whereas OMD depends on OM digestion in the total digestive tract. A reduced degradation in the rumen may be compensated by enhanced fermentation in the hindgut. Therefore, differences between results of these alternative methods will probably be more pronounced when correlated with *in vivo* FOM than with *in vivo* OMD. Reproducibility of enzymatic methods is generally higher than of methods using rumen fluid, like with the *in situ* method and some other *in vitro* techniques. Compared with *in vitro* methods that use rumen fluid, the method of Tilley & Terry (1963) is not dynamic and therefore has less variable results than the gas production technique.

Alternative methods most widely used in France and the Netherlands are the pepsin-cellulase (Aufrère & Demarquilly, 1989), the *in situ* (Michalet-Doreau *et al.*, 1987), the gas production (Cone *et al.*, 1996) and the *in vitro* technique of Tilley & Terry (1963). In our study, these four methods were evaluated for their suitability to estimate *in vivo* FOM. For this estimation, alternative DOM was related directly to *in vivo* FOM and indirectly to *in vivo* FOM using the calculations from the French and Dutch protein evaluation systems (Vérité *et al.*, 1987; Tamminga *et al.*, 1994).

The main objective of this study was to determine whether these calculations improve the accuracy of the *in vivo* FOM estimate, and which alternative method estimates *in vivo* FOM most accurately.

Materials and methods

Forages

OM digested in the rumen and OM digested in the total digestive tract were determined for 12 forage types including the fresh form, silage and hay of lucerne (*Medicago sativa*), red clover (*Trifolium pratense*), orchard grass (*Dactylis glomerata*) and perennial ryegrass (*Lolium perenne*). Because of wet harvesting conditions red clover hay was substituted by red clover haylage, a baled wilted forage stored in sealed plastic wraps, with a dry matter content of about 500 g per kg forage.

In vivo measurement of organic matter degradation

In vivo FOM was measured as organic matter truly digested in the rumen (OMTDR) using fistulated sheep. OMTDR is the sum of OM apparently digested in the rumen and bacterial OM synthesized in the rumen and entering the duodenum. OM apparently digested in the rumen is the difference between OM intake and OM entering the duodenum. Bacterial OM entering the duodenum was calculated from the duodenal flow of bacterial nitrogen (N) assuming a N/OM ratio in the bacteria of 1:10 (Clark *et al.*, 1992).

OM duodenal flow, bacterial N and organic matter total tract digestibility (OMD) were measured *in vivo* in an experiment using the methodology described by Rémond *et al.* (2003). The experiment comprised six cannulated sheep fed restricted (90% of *ad libitum*), and used ^{51}Cr-EDTA and ^{103}Ru-Phenanthrolin as flow markers and ^{15}N as microbial marker.

The same methodology was used for the grass and the legume forages, with the exception of fresh perennial ryegrass for which only the flow marker ^{103}Ru-Phenantrolin (non-radioactive) was used. Comparing the single-marker with the double-marker results for the other 11 forages showed that the difference in duodenal flow of OM and non-ammonia N was not statistically significant. But as the bacterial N flow was about 5.4% (range 0.75–8%) lower with the single marker, the duodenal flow of bacterial OM for fresh perennial ryegrass was increased with 5.4%.

In situ and *in vitro* measurement of dry and organic matter degradation

One *in situ* (Michalet-Doreau *et al.*, 1987) and three *in vitro* methods were used for measuring OM and dry matter (DM) degradation of the 12 forages. The *in vitro* methods were the pepsin-cellulase (Aufrère & Demarquilly, 1989), the gas production

(Cone *et al.*, 1996) and the two-stage *in vitro* technique of Tilley & Terry (1963). These methods were used to estimate *in vivo* FOM directly and indirectly.

In situ *method*

The method of sample preparation for the *in situ* measurement (nylon bag technique) of DM degradation has been described by Dulphy *et al.* (1999). The procedure of the measurement was according to Michalet-Doreau *et al.* (1987) and the data were fitted according to Ørskov & McDonald (1979). Effective degradable DM was calculated using different passage rates. A passage rate of DM in the total tract of 3% h^{-1} gave best results for estimating OMD (Gosselink *et al.*, 2004). A ruminal passage rate of DM of 4.5% h^{-1} is used in the Netherlands (Tamminga *et al.*, 1994) and 6% h^{-1} in France (Vérité *et al.*, 1987). In our calculations also a passage rate (kp) equal to rumen degradation rate (kd) was used. kp as function of kd improved the estimate of FOM as calculated in the Dutch protein evaluation system (Van Vuuren, 1993).

Two incubation series were carried out per forage type. The two series were incubated at the beginning and at the end of the week. Each forage type was incubated in three cows that were fed a ration of 70% forage and 30% concentrates.

Pepsin-cellulase technique

The pepsin-cellulase technique developed by Aufrère (1982) is an enzymatic method for measuring DM degradation. It includes the use of 0.1 N HCl (Aufrère & Demarquilly, 1989). DM degradation of each forage type was determined in triplicate.

In vitro *method according to Tilley & Terry*

OM digestibility was determined with the two-stage *in vitro* method using rumen fluid and acid pepsin as described by Tilley & Terry (1963). In our comparative study, measured values and values standardized with *in vivo* values according to the modification of Van Der Meer (1986) were used. The measured and standardized values were determined in duplicate.

Gas production technique

The forages were incubated in quadruplicate, using the gas production technique as described by Cone *et al.* (1996). Gas production profiles were analysed with a three-phase model (Groot *et al.*, 1996), describing the gas production caused by fermentation of the soluble components (phase 1), the non-soluble components (phase 2) and the microbial turnover (phase 3) (Cone *et al.*, 1997). Each phase is described with the parameters a, b and c; a: maximum gas production, ml per g OM, b: time in hours needed to reach 50% of the maximum gas production, and c: dimensionless parameter determining the shape of the curve.

After 72 hours of incubation also OM degradation (as % of OM incubated) was determined by measuring the OM residue after filtering over a P1 glass crucible.

Calculation of fermentable organic matter

Fermentable organic matter (FOM) was calculated from OMD according to the French and Dutch protein evaluation systems (FFOM and DFOM, respectively; Vérité *et al.*, 1987; Tamminga *et al.*, 1994). FFOM and DFOM were used for the indirect estimate of *in vivo* FOM. Different origins of OMD were used: OMD measured *in vivo* and OMD estimated with results from the alternative methods (Gosselink *et al.*, 2004). To calculate FFOM and DFOM from OMD, the amounts of fermentation products (silages and haylage), rumen escape protein and crude fat from forages were subtracted from OMD. Different proportions of fermentation products in silage and haylage caused differences between FFOM and DFOM. In the French system 100% and in the Dutch system 50% of the amount of fermentation products was taken into account for calculating FFOM and DFOM, respectively. Fermentation products were determined according to Dulphy *et al.* (1975). Rumen escape protein of the 12 forages was measured using the *in situ* method described in this paper and calculated as in Michalet-Doreau & Ould-Bah (1989). It was assumed that the forages contained no starch and that crude fat content was 15 g per kg OM for hay and 30 g per kg OM for the other forages.

FFOM and DFOM (g per kg OM intake) were calculated from crude protein (CP) and from OMD (g per kg OM intake) estimated with equations developed by Gosselink *et al.* (2004), using the *in situ*, the pepsin-cellulase, the Tilley & Terry and the gas production technique.

For the results from the *in situ* technique and CP, the equation was:

$$OMD = 275 + 0.696 \times \text{effective DM degradation} - 0.621CP$$

for the results from the pepsin-cellulase technique and CP:

$$OMD = 394 + 0.512 \times \text{DM degradation} - 0.484CP$$

for the results from the technique of Tilley & Terry:

$$OMD = 0.966 \times \text{OM degradation (measured } \textit{in vitro} \text{ values)}$$

and for the results from the gas production technique and CP:

$$OMD = 300 + 1.162 \times \text{gas production after 20 hours} + 0.332CP$$

Chemical analysis

DM contents of feed and residues in the nylon bags were determined by drying at 80 °C for 48 hours. Ash content was determined after 6 hours at 550 °C. DM content of silage and haylage was corrected for fermentation products (Dulphy *et al.*, 1975). N was determined with the Kjeldahl method (Anon., 1980). Neutral detergent fibre

(NDF) and acid detergent fibre (ADF) were determined in the samples dried at 80 °C, using the method described by Van Soest *et al.* (1991).

Statistical analysis

The statistical analyses were carried out with Genstat (Anon., 2002). To improve the direct estimate of *in vivo* FOM from the alternative techniques and the indirect estimates from FFOM and DFOM, the factors forage family (legume or grass) and method of conservation (fresh or conserved) and the covariable chemical components were included in the analyses. The following model equation was used to estimate *in vivo* FOM:

$$\textit{In vivo}\ \text{FOM} = \beta_0 + \beta_1 \times \text{technique} + \beta_2 \times \text{covariable} + \text{factor} + \varepsilon$$

where

technique = FFOM and DFOM, or DM or OM degradation measured by the pepsin-cellulase, the *in situ*, the gas production or the Tilley & Terry technique,
covariable = chemical components,
factor = forage family (legume or grass) or method of conservation (fresh or conserved),
β_1 and β_2 = regression coefficients, and
ε = residual error.

The estimates of *in vivo* FOM were considered statistically significant if $P < 0.05$.

To evaluate the estimates of *in vivo* FOM, their R^2 and RSE (residual standard error) values were compared. If equations with intercept ≠ 0 and equations with intercept = 0 had similar R^2 and RSE values, the equation with intercept = 0 was chosen for its simplicity.

The mean square prediction errors (MSPE) of the estimates of *in vivo* FOM were compared. MSPE was calculated from the differences between the observed and the predicted values, using the following equation (Bibby & Toutenberg, 1977):

$$\text{MSPE} = 1/n \sum(O - P)^2$$

where

O = the observed value,
P = the estimated value, and
n = number of observations.

The square root of MSPE expressed as percentage of the observed mean was used as a measure of the prediction error (PError). MSPE was split up in error in central tendency (bias), error due to the regression slope deviating from 1 and error due to disturbances (unexplained variation) (Bibby & Toutenberg, 1977).

Results

General

The large variation in quality of the 12 forage types (Tables 1 and 2) resulted in a large range of data on OM digested in the rumen or in the total tract, measured *in vivo* (Table 3), and thus facilitated obtaining estimates of *in vivo* FOM.

The use of different forages also resulted in a large range of data on degraded OM and DM measured *in situ* and *in vitro* (Table 4). The standard deviation (SD) of the results per type of forage was higher with the *in situ* and the gas production technique than with the other methods. The average coefficients of variation for the data obtained *in situ* were lower (3.7) and for the data obtained with the gas production technique after 20 hours (gp20) were higher (6.0) than those obtained for OMTDR (Tables 3 and 4).

As FFOM and DFOM were calculated from OMD, the variation in FFOM and DFOM values (Table 5) and their SD depended on the method used for measuring OMD. OMD was either measured *in vivo* (Table 3) or was estimated with alternative methods (Table 4).

Table 1. Dry matter (DM) content, chemical composition and rumen escape protein (REP) determined with the *in situ* technique, for the 12 types of forage studied.

Forage	State of conservation	DM	Chemical composition[1]				REP
			Ash	CP	NDF	ADF	
		(g kg^{-1})	- - - - - - - - - - (g per kg DM) - - - - - - - - - -				
Lucerne	Fresh	162	138	198	498	346	43.4
	Silage	212	98	182	438	328	32.2
	Hay	861	99	171	560	379	54.0
Red clover	Fresh	127	120	168	492	348	18.5
	Silage	171	92	166	478	343	28.3
	Haylage	524	108	128	475	352	25.9
Orchard grass	Fresh	193	80	116	676	360	33.9
	Silage	217	71	126	614	343	20.4
	Hay	852	70	110	697	376	36.7
Perennial	Fresh	182	98	91	620	366	17.5
ryegrass	Silage	191	92	101	578	371	11.8
	Hay	873	96	91	632	382	25.5

[1] CP = crude protein; NDF = neutral detergent fibre; ADF = acid detergent fibre.

Table 2. Chemical composition[1] of the 4 silages and 1 haylage from Table 1.

Forage	pH	NH_3	HL	HAc	HP	HB	Ethanol
		(g per kg DM)					
Silage							
Lucerne	4.03	2.24	45.6	29.6	0.30	0	5.79
Red clover	3.97	1.93	69.4	23.6	0.73	0.20	4.08
Orchard grass	3.93	1.21	78.5	14.9	0.15	3.09	3.42
Perennial ryegrass	4.13	0.66	92.9	19.1	2.15	0.40	17.5
Haylage							
Red clover	5.11	1.71	24.3	3.6	0.96	0.75	2.35

[1] HL = lactic acid; HAc = acetic acid; HP = propionic acid; HB = butyric acid.

Table 3. Organic matter intake (OMI), apparently digested organic matter in the rumen (OMADR), truly digested organic matter in the rumen (OMTDR) and organic matter digested in the total tract (OMD), measured *in vivo* in sheep. Means and standard deviations (in parentheses) for the 12 types of forage from Table 1.

Forage	State of conservation	OMI	OMADR	OMTDR	OMD
		g day^{-1}	(g per kg OM intake)		
Lucerne	Fresh	1329	329	553 (27.7)	592 (17.9)
	Silage	1519	410	583 (10.3)	641 (9.3)
	Hay	1028	346	523 (33.1)	559 (10.0)
Red clover	Fresh	1141	518	739 (31.6)	725 (16.7)
	Silage	1206	458	624 (24.5)	682 (13.0)
	Haylage	1148	447	617 (29.2)	646 (15.7)
Orchard grass	Fresh	1226	419	609 (17.6)	629 (8.3)
	Silage	1214	383	556 (27.7)	612 (16.9)
	Hay	1078	357	519 (13.2)	558 (22.5)
Perennial ryegrass	Fresh	1191	519	691 (23.5)	671 (15.2)
	Silage	1195	420	609 (23.5)	658 (14.2)
	Hay	1162	407	589 (27.9)	635 (5.4)

Table 4. Dry matter (DM) degradation determined with the *in situ* technique in cows (In situ) and with the pepsin-cellulase technique (Pep-Cel), organic matter (OM) degradation measured with the Tilley & Terry method (T&T) and with the gas production technique (Gpdeg), and the gas production after 20 hours of incubation (Gp20). Means with standard deviations (SD) for the 12 types of forage from Table 1.

Forage	State of conservation	DM degradation				OM degradation				Gp20	
		In situ		Pep-Cel		T&T		Gpdeg		Mean	SD
		Mean	SD	Mean	SD	Mean	SD	Mean	SD		
		– (g per kg DM intake) – –				– (g per kg OM intake) – –				(ml per g OM)	
Lucerne	Fresh	589	21.5	631	4.4	626	3.5	643	5.0	164	6.6
	Silage	593	10.6	640	2.8	680	0.7	687	4.6	182	6.2
	Hay	484	8.5	605	4.5	606	0.7	625	5.0	159	10.4
Red clover	Fresh	709	14.2	696	7.1	700	2.1	761	4.5	218	8.0
	Silage	639	12.1	649	3.3	675	0	718	5.0	203	10.1
	Haylage	573	24.7	668	4.0	690	10.6	732	2.2	209	21.2
Orchard grass	Fresh	491	33.0	516	3.8	642	1.4	696	6.4	183	14.1
	Silage	496	6.7	556	2.8	609	1.4	714	4.4	188	9.8
	Hay	407	16.8	462	2.9	672	1.4	661	3.3	165	10.5
Perennial	Fresh	532	23.5	587	3.3	728	1.4	766	8.3	222	16.6
ryegrass	Silage	551	49.7	583	6.2	684	3.5	727	4.0	210	7.6
	Hay	506	16.8	546	8.7	673	6.3	701	3.0	192	17.4

Directly estimated *in vivo* FOM

Of all methods used for directly estimating *in vivo* FOM, the gas production technique gave best results (Table 6). Gas production after 20 hours of incubation (gp20), whether corrected for CP or not, was well correlated with *in vivo* FOM. The highest R^2 and lowest RSE values were found when the relationship between *in vivo* FOM and gp20 was separated in relationships for fresh and conserved forages. But the relationship did not improve when other parameters from the gas production profiles were included. Therefore, only gp20 is presented in the tables.

High R^2 and low RSE values for the relation between estimated *in vivo* FOM and the results from the *in situ* technique were also found if CP was included. If excluded the best results were obtained if kp was 3% h^{-1} or if kp = kd (Table 6). The relationship between the OM degraded after 72 hours of incubation (gp72) in the gas production method, the results from the pepsin-cellulase technique, and the results with the method of Tilley & Terry on the one hand and estimated *in vivo* FOM on the other were similar. However, in these relations MSPE was partly due to regression and not to general disturbance.

Table 5. Mean fermentable organic matter (OM) calculated for the 12 types of forages from Table 1 with the French (FFOM) and the Dutch (DFOM) protein evaluation methods, using organic matter total tract digestibility (OMD) measured *in vivo* (In vivo) and OMD predicted with the *in situ* technique (In situ), the pepsin-cellulase technique (Pep-Cel), the method of Tilley & Terry (T&T) or the gas production technique (GPT).

Forage	State of conservation	Protein evaluation method	Method to estimate OMD				
			In vivo	In situ	Pep-Cel	T&T	GPT
			(g per kg OM intake)				
Lucerne	Fresh	FFOM&DFOM	512	521	542	509	530
	Silage	FFOM	496	471	489	480	502
		DFOM	536	511	528	520	542
	Hay	FFOM&DFOM	483	484	545	499	519
Red clover	Fresh	FFOM&DFOM	674	648	618	617	641
	Silage	FFOM	476	493	476	479	502
		DFOM	530	547	531	534	557
	Haylage	FFOM	587	553	579	554	577
		DFOM	605	571	597	572	595
Orchard grass	Fresh	FFOM&DFOM	562	547	535	532	553
	Silage	FFOM	451	451	456	445	466
		DFOM	505	506	511	499	521
	Hay	FFOM&DFOM	504	514	523	512	532
Perennial ryegrass	Fresh	FFOM&DFOM	622	605	601	614	637
	Silage	FFOM	469	466	455	458	481
		DFOM	541	538	527	530	553
	Hay	FFOM&DFOM	591	592	586	576	598

No single chemical component was significantly related to *in vivo* FOM. Combinations of chemical components or including DM or ash as variables, did not improve the relationships between chemical components and *in vivo* FOM.

Indirectly estimated *in vivo* FOM

Generally, comparing R^2, RSE and the contribution of the regression to MSPE (Table 7), *in vivo* FOM was better estimated with DFOM than with FFOM. Although there was little difference between the values obtained with DFOM and FFOM, they were lower than *in vivo* FOM measured in fistulated animals.

When FFOM and DFOM were calculated from OMD estimated *in situ* or *in vitro*, R^2 was lower and RSE higher than when *in vivo* determined OMD values were used. The *in situ* technique resulted in the best indirect estimate of *in vivo* FOM, but also the gas production technique and the method of Tilley & Terry gave good results.

Table 6. Regression equations for directly estimating *in vivo* fermentable organic matter (Y; g *in vivo* FOM per kg OM intake) in sheep from dry matter (DM) or OM degradation determined with the methods in Table 4, including or excluding crude protein (CP) and assuming different conditions.

Method/ variables	Equation[a]	R^2	RSE[b]	MSPE[c] due to		
				Regression	Bias	Disturbance
				(%)		
In situ						
Kp[d] = 3.0	0.955X	0.56	43	6.4	0	93.6
+ CP	1.160X – 0.937CP	0.78	30	7.7	0.1	92.2
kp = 4.5	1.027X	0.31	53	29.6	0.4	70.0
	233 + 0.632X	0.47	47	0	0	100
+ CP	220 + 0.912X – 1.090CP	0.78	30	0	0	100
kp = 6.0	1.027X	< 0.1	63	45.0	0.9	54.1
	295 + 0.560X	0.43	49	0	0	100
+ CP	293 + 0.856X – 1.176CP	0.78	30	0	0	100
kp = kd[e]	1.094X	0.59	41	2.2	0	97.8
Pepsin-cellulase						
	1.004X	–	65	27.7	0.3	72.0
Tilley & Terry						
Measured values	0.905X	0.48	46	7.8	0.1	92.1
Standardized values	0.903X	0.59	40	20.1	0	79.9
Gas production technique						
OM degradation after 72 hours	0.857X	0.67	37	26.2	0.1	73.7
Gas production after 20 hours	3.139X	0.74	33	11.4	0.1	88.5
+ MC[f]	176 + 2.406X (fresh)	0.90	21	0	0	100
	124 + 2.406X (conserved)					
+ CP	2.815X + 0.418CP		29	0.5	4.7	94.8

[a] X = variable depending on method used.

[b] RSE = residual error.

[c] MSPE = mean square prediction error.

[d] kp = passage rate.

[e] kd = rumen degradation rate.

[f] MC = method of conservation: fresh or conserved forage.

Table 7. Regression equations for indirectly estimating *in vivo* fermentable organic matter (*in vivo* FOM; g per kg OM intake) in sheep, from fermentable organic matter (FOM) calculated with the French or the Dutch procedure (Table 5; FFOM and DFOM, respectively). FOM calculated from OMD (digested OM in the total tract) measured *in vivo* in sheep (vivo) and from OMD predicted with the *in situ* technique (situ), the pepsin-cellulase technique (p-cel), the method of Tilley & Terry (T&T) or the gas production technique (gpt).

Variable (X)	Equation	R^2	RSE[a]	MSPE[b] due to		
				Regression	Bias	Disturbance
				(%)		
FFOM-vivo	1.116X	0.36	51	33.2	0.4	66.4
	22.8 + 0.696X	0.53	44			100
DFOM-vivo	1.081X	0.82	27	1.6		98.4
FFOM-situ	1.132X	0.38	51	24.2	0.3	75.6
DFOM-situ	1.096X	0.80	29	2.4		97.6
FFOM-p-cel	1.121X	–	64	25.0	0.3	74.7
DFOM-p-cel	1.086X	0.52	45	1.8		98.2
FFOM-T&T	1.145X	0.34	53	20.3	0.2	79.5
DFOM-T&T	1.109X	0.76	32	7.3		92.7
FFOM-gpt	1.075X	0.33	52	11.8	0.3	87.9
DFOM-gpt	1.042X	0.74	32	25.3		74.7

[a] RSE = residual error.

[b] MSPE = mean square prediction error.

In vivo FOM indirectly estimated using OMD values measured *in situ* or *in vitro*, was close to *in vivo* FOM directly estimated with these methods. Moreover, with the method of Tilley & Terry, the indirect estimate of *in vivo* FOM had a higher R^2 and a lower RSE than the direct estimate.

Discussion

General

Because of methodology, costs and animal welfare it is more difficult to measure *in vivo* FOM than *in vivo* OMD. The variation of *in vivo* FOM is larger and the result of measuring *in vivo* FOM is also less precise. Moreover, OMD estimates from *in vitro* and *in situ* techniques are well validated (Gosselink *et al.*, 2004). Our results showed that the direct estimate of *in vivo* FOM was slightly superior to the indirect one.

Directly estimated *in vivo* FOM

In vivo FOM estimated with the gas production technique and the *in situ* technique improved when a correction for CP was made. In the gas production technique protein fermentation influences gas production negatively (Cone & Van Gelder, 1999; Chenost *et al.*, 2001). Especially with the *in situ* technique accuracy was considerably improved when CP content was included. *In situ* measurement of OM and DM degradation includes all CP degraded in the rumen, whereas *in vivo* FOM does not include CP degraded to ammonia entering the duodenum. The regression coefficient of CP increased with increasing kp. The CP fraction in the equation probably corrects for the difference in degradable CP (or for other OM fractions flowing out of the rumen) between *in vivo* FOM and effective degradable DM measured with the *in situ* technique.

The difference in *in vivo* FOM estimated with the gas production technique between fresh and conserved forages was a result of differences in digestibility. Silage has a lower soluble carbohydrate content than fresh forage and the structural carbohydrate composition of hay can be affected by leaf losses during harvesting (Merchen & Bourquin, 1994).

Indirectly estimated *in vivo* FOM

In vivo FOM was better estimated using 50% of fermentation products for the calculation of DFOM than using 100% of fermentation products to calculate FFOM, although both resulted in an underestimation of *in vivo* FOM.

The best OMD estimates from alternative methods, reported by Gosselink *et al.* (2004) and used in this study, took CP as covariable into account, except in the case of the Tilley & Terry method.

Rumen digestion and alternative methods

Rumen digestion dynamics are important in both the direct and the indirect method of estimating *in vivo* FOM. Ruminal OM degradation is part of the rumen digestion dynamics mimicked by the *in situ* and the *in vitro* methods. Another important part is the ruminal OM passage rate. Only estimates based on results from the *in situ* technique take passage rate into account, although feed evaluation systems use a constant

rumen passage rate (Vérité *et al.*, 1987; Tamminga *et al.*, 1994). With a variable passage rate (kp = kd), the *in vivo* FOM estimate improved when based on results from the *in situ* technique. However, when passage rates were varied the effect on *in vivo* FOM was small. This was mainly the result of the limited effect of DM intake on ruminal passage rates and OM digestion when forages are fed above maintenance (Galyean & Owens, 1991; Chilliard *et al.*, 1995).

Ranking the alternative methods

The order in which the alternative methods estimate OMD most accurately, as observed by Gosselink *et al.* (2004), i.e., *in situ* technique ≤ gas production technique ≤ method of Tilley & Terry ≤ pepsin-cellulase technique, was similar to the order we found for the directly and indirectly estimated *in vivo* FOM values. It was also similar to the order in which the results of the techniques most closely approached the rumen digestion dynamics. However, this order was found without taking into account the influence of a covariable (like CP) or a factor (like method of conservation) for the accuracy of a prediction equation. A covariable contributes to the explanation of the variation and thus reduces RSE (Table 6). However an equation with a covariable is likely to have a higher RSE value than an equation without this, because a second determinant in the equation will decrease reproducibility as each determination has its inaccuracy. When only one variable was used to directly estimate *in vivo* FOM, the gas production technique had lowest RSE and highest R^2. Of the alternative methods used for indirectly estimating *in vivo* FOM, only the method of Tilley & Terry (*in vitro* values) did not include a covariable.

To discriminate between estimates a threshold for accuracy is set by assuming a limit for RSE. RSE should be lower than 5% of the mean *in vivo* FOM (600 g kg^{-1}) and only prediction equations with a RSE lower than 30 should be used. So only *in vivo* FOM indirectly estimated from results with the *in situ* technique using the DFOM calculation method, and *in vivo* FOM directly estimated from gp20 results separated for fresh and conserved forages should be chosen. Nevertheless, *in vivo* FOM indirectly estimated from the *in situ* technique using calculated DFOM values had a high R^2. This indirectly estimated *in vivo* FOM is kind of a validation, because the calculation of DFOM values from degradable OM measured with the *in situ* technique was validated in another study (Gosselink *et al.*, 2004). The regression for directly estimating *in vivo* FOM from the results of gas production after 20 hours had a high R^2 value, especially when fresh and conserved forages were separated (Table 6).

A disadvantage of the *in situ* and the gas production techniques is the large variation of the results and thus the low reproducibility of these methods. So these alternative methods need more repetitions than the more static alternative *in vitro* methods that use enzymes or chemicals.

The *in situ* method used in this study probably had another disadvantage, because the nylon bags were incubated in the rumen of cows whereas *in vivo* FOM was determined in sheep. Caution is needed when extrapolating the results from one animal species to the other, as different species can differ in ruminal passage rates, feed digestibility (Colucci *et al.*, 1990; Dulphy *et al.*, 1994; Poncet *et al.*, 1995) and degrada-

tion characteristics (Šebek & Everts, 1999). Nevertheless, a good relationship between effective degradable DM and *in vivo* FOM was observed in this study.

The choice of an alternative *in situ* or *in vitro* technique will also depend on costs, time, experience, animal welfare and availability of *in vivo* FOM data to validate the estimates. The additional information resulting from an alternative method will be important too, especially the information on rumen dynamics. The *in situ* and the gas production technique also yield rates of degradation or fermentation of OM and the *in situ* technique can provide degradation rates of other nutrients.

Conclusion

In vivo FOM was best indirectly estimated using the calculation from the Dutch protein evaluation system. The indirect estimate was more accurate with the results of the *in situ* and the gas production techniques, i.e., the most dynamic methods for measuring OM degradation, than when the other *in vitro* methods were used.

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