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Use of *in vivo*, *in situ* and *in vitro* Gas Production Techniques to Evaluate Nutritive Value of Alfalfa Hay in Sheep

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Abstract: The nutritive value of alfalfa (variety of Hamedani) was evaluated at late maturity (mid to late bloom). The *in vivo* digestibility of the hay was determined by conducting digestibility and respiration trials in three Gezel rams. Dry matter, organic matter and protein degradability of the hay was determined by nylon bag technique and *in vitro* Organic Matter Digestibility (OMD) and Metabolizable Energy (ME) content were determined by Gas production technique. The results showed that the crude protein, Neutral Detergent Fiber (NDF) and Acid Detergent Fiber (ADF) content of alfalfa hay were 15.8, 43.1 and 29.4%, respectively. *In vivo* Dry Matter (DMD), Organic Matter (OMD), Crude Protein (CPD) digestibilities and ME content in alfalfa hay were 65, 66.7, 64% and 9.6 MJ per kg DM, respectively. *In situ* DM, OM and CP effective degradability at a rate 0.02 h^{-1} were 64, 61.23 and 49.6%, respectively. Gas production technique OMD and ME content were 71.2% and 10.69 MJ per kg DM. Results obtained with nylon bag technique were confirmed by the *in vivo* experiments involving the alfalfa hay samples.

Key words: *In vivo*, *In situ*, *in vitro* gas production, alfalfa, sheep

INTRODUCTION

Alfalfa, harvested as hay, is an important forage crop for lactating cows because of its high protein concentration; however, research has indicated that the proteins in alfalfa are highly susceptible to degradation during field-wilting (Makoni *et al*., 1993; Mangan *et al*., 1991; Papadopoulos *et al*., 1983) and ruminal fermentation (Broderick, 1985). Nutritive value of forages depends on their dry matter digestibility and voluntary dry matter intake (Kamalak *et al*., 2005).

The determination of forage intake and digestibility by using *in vivo* methods is time-consuming, laborious and expensive and requires a large quantity of forage. It is also unsuitable for large-scale feed evaluation laboratories. The nylon bag technique has been used for many years to provide a useful means for estimating rates of disappearance and potential degradability of feed-stuffs (Ørskov and Ryle, 1990; Chermiti *et al*., 1996). This technique also provides an opportunity to fractionate feedstuffs into water soluble, potentially degradable and indigestible fractions, which gives some idea about the extent of degradation of feedstuffs in the rumen (Ørskov and Ryle, 1990). The *in vitro* gas production technique has proved to be a potentially useful technique for feed evaluation (Menke and Steingass, 1988; Blummel and Ørskov, 1993; Herrero *et al*., 1996; Getachew *et al*., 2004), as it is capable of measuring rate and extent of nutrient degradation (Groot *et al*., 1996; Cone *et al*., 1997). In addition, *in vitro* gas production technique provide less expensive (Getachew *et al*., 2004), easily to determine (Khazaal *et al*., 1993) and suitable for use in developing countries (Blummel *et al*., 1997).

The aim of the present study, was to determine chemical composition and potential nutritive value alfalfa hay by using *in vivo*, *In situ* and *in vitro* gas production techniques.

MATERIALS AND METHODS

Forage: Alfalfa (variety of Hamedani) was used in the experiment. Samples of the alfalfa were collected randomly from ten farms in West Azerbaijan, Iran and evaluated at the laboratories of Animal Science Research Institute in Karaj and at the laboratories of Islamic Azad University-Shabestar Branch.

Forage, at harvested, was estimated to be at late maturity (mid to late bloom). Samples were collected, air-dried and ground (1mm and 5mm screen) for chemical analysis, *In situ* and *in vitro* gas production.

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Chemical analysis: Dry Matter (DM) was determined by drying the samples at 105°C overnight and ash by igniting the samples in muffle furnace at 525°C for 8 h and Nitrogen (N) content was measured by the Kjeldahl method (AOAC, 1990). Crude Protein (CP) was calculated as N×6.25. Neutral Detergent Fiber (NDF), Acid Detergent Fiber (ADF), Acid-Detergent Lignin (ADL) and Acid Insoluble Ash (AIA) were determined by procedures outlined by Goering and VanSoest (1970) with modifications described by VanSoest *et al.* (1991); sulfite was omitted from NDF analysis. Hemicellulose and cellulose were calculated as (NDF-ADF) and (ADF-ADL-AIA), respectively (Andrighetto *et al.*, 1993).

***In vivo* digestibility trial:** Alfalfa was offered ad libitum to three Gezel rams (1.5 year old, avg initial BW 55 kg) kept in metabolism cages to enable accurate determination of feed intake and allow easy collection of faeces. The forage was fed twice daily at 08:30 and 16:30 h and fresh drinking water and mineral salt licks were freely available. The animals were adapted forage for two weeks, followed by balance trails of seven days, in which daily measurement of food intake and fecal excretion were made. Sub-sample of forage was taken and data on their daily intake (g DM kg^{-1} $LW^{0.75}$) and digestibility *in vivo* were obtained.

***In situ* degradation procedures:** Three 55 kg ruminally cannulated Gezel rams were used to determine *In situ* degradation characteristics. Rams were housed in individual tie stalls bedded with sawdust. Rams fed chopped alfalfa hay containing 14% CP and 45% NDF were used for incubation of samples in Dacron bags in this study. Alfalfa hay was offered 1.25×maintenance levels of rams (Karsli *et al.*, 2002).

In situ procedures were the same as those described previously by Coblentz *et al.* (1997); Dacron bags (18×9 cm; 520 mm pore size) were filled with 5-g samples of dried ground forage. Suspension of bags in the rumen was accomplished by tying of bags, into tygon tubing with nylon string. Sample in Dacron bags were placed in the rumen of rams and incubated for the periods of 0, 4, 8, 16, 24, 48, 72 h. After the removal of bags from the rumen, bags were washed in cold water until rinse were clear and dried at 60°C for 48 h (Karsli *et al.*, 2002). Remaining residues were analyzed for DM, OM and N concentrations.

Dry matter, organic matter and N were divided into three fraction as follows: The soluble DM, OM or N fraction (fraction a) determined as DM, OM or N loss during the washing process, The potentially digestible DM, OM or N fraction (fraction b) determined as the differences between initial DM, OM or N content after washing and the amounts of DM, OM or N recovered after a 72 h incubation, The indigestible fraction (fraction c) determined as the amount of DM, OM or N residue recovered after a 72 h incubation (Karsli *et al.*, 2002).

Rumen degradation kinetics for DM, OM and CP were calculated using the nonlinear model proposed by Ørskov and McDonald (1979):

$$P = a+b*(1-e^{c*t})$$

P = Percentage of degradability for response variables at t
t = Time relative to incubation (h).
a = Highly soluble and readily degradable fraction (%).
b = Insoluble and slowly degradable fraction (%).
c = Rate constant for degradation (% h^{-1}).

Following determination of these parameters, the effective degradability of nutrients in alfalfa hay was calculated using and equation described by Ørskov and McDonald (1979):

$$Pe = a+(b*c)/(c+k)$$

Pe = Effective degradability for response variables (%).
a = Highly soluble and readily degradable fraction (%).
b = Insoluble and slowly degradable fraction (%).
c = Rate constant for degradation (% h^{-1}).
k = Rate constant of passage (h^{-1}).

When calculating effective degradability, rate constant of passage was assumed to be 0.02% per hour (Bhargava *et al.*, 1987) so that the results could be extrapolated to other ruminants that differ in rumen capacity.

DM degradation *In situ* (g DM $kg^{-1}W^{0.75}$) = 9.9+0.40 (a+b)+408(c) (Khazaal *et al.*, 1995).

***In vitro* gas production:** Fermentation of alfalfa hay samples were carried out with rumen fluid obtained from three fistulated Gezel rams (1.5 year old, avg initial BW 55 kg) fed twice daily with a diet containing hay (60%) and concentrate (40%) following the method described by Menke and Steingass (1988). Approximately 200 mg hay samples were weighed into the glass syringes of 100 mL. The fluid-buffer mixture (30 mL) was transferred into the glass syringes of 100 mL. The glass syringes containing hay samples and rumen fluid-buffer mixture were incubated at 39°C. The syringes were gently shaken 30 min after the start of incubation. The gas production was determined after 2, 4, 6, 8, 12, 24, 48, 72 and 96 h of incubation. All samples were incubated in triplicate with

3 syringes containing only rumen fluid-buffer mixture (blank). The net gas productions for hay samples were determined by subtracting the volume of gas produced in the blanks. Gas production data were fitted to the model of Ørskov and McDonald (1979).

$$Y = a+b\ (1-e^{-ct})$$

Where,

a = The gas production from the immediately soluble fraction (mL)
b = The gas production from the immediately insoluble fraction (mL)
c = The gas production rate constant for the insoluble fraction (% h^{-1})
a+b = Potential gas production (mL)
t = Incubation time (h)
Y = Gas production at time t

The ME (MJ per Kg DM) contents of alfalfa hay samples were calculated using equation of Menke *et al.* (1979) as follows:

$$ME\ (MJ\ per\ Kg\ DM) = 2.20+0.136\ GP+0.057\ CP$$

Where
GP = 24 h net gas production (mL 200 mg^{-1}).
CP = Crude protein (%)

Organic Matter Digestibility (OMD) (%) of alfalfa hay samples were calculated using equation of Menke *et al.* (1979) as follows:

$$OMD\ (\%) = 14.88+0.889\ GP+0.45\ CP+0.0651\ XA$$

Where
GP = 24 h net gas production (mL 200 mg^{-1}).
CP = Crude protein (%)
XA = Ash content (%)

$$DMI\ (g\ DM\ kg^{-1}W^{0.75}) = -2.6+0.49(a+b)+339(c)+0.17\ (CP)$$

$$DMD\ (g\ kg^{-1}\ DM) = 8.3(a+b)+190\ (Khazall\ \textit{et al.}, 1995)$$

In vitro gas production measurements were carried out in the laboratory of Animal Science Research Institute in Karaj.

RESULTS AND DISCUSSION

Chemical analysis: The chemical compositions of forage are presented in Table 1. The CF content of alfalfa hay was 29.2%. On the other hand ADF and NDF contents ranged from 29.4±0.8 to 34.4±0.2. The cell wall (ADF and NDF) and ADL contents of HAM hay were similar than those reported by Coblentz *et al.* (1998), lower than that reported by Torrent *et al.* (1994) and higher than that reported by Kamalak *et al.* (2005a). The different result reported by several researchers about alfalfa hay cell wall content may be due to differences in maturity (Coblenze *et al.*, 1998; Gulsen *et al.*, 2004; Kamalak *et al.*, 2005b, c) variety, environmental conditions and agronomic factors (Wechsler, 1981; Buxton, 1996) and leaves to stem ratio (Coblenze *et al.*, 1998). It is well established that the cell wall content of forages increase with increasing maturity (Gulsen *et al.*, 2004; Kamalak *et al.*, 2005b, c). The GE content for alfalfa hay was 4219 Kcal Kg^{-1}. The CP content of forage hay (15.8%) was lower than that

Table 1: Chemical composition of alfalfa hay

Component	alfalfa	SE
DM, %	92.93	0.265
OM, %	89.66	0.245
	----------(%DM)----------	
CP	15.8	0.828
Crude fiber	29.2	0.508
Ether extract	1.33	0.193
Ash	10.33	0.245
NFC	29.44	0.677
NFE	43.3	0.790
Cell contents	56.9	0.307
NDF	43.1	0.307
ADF	29.4	0.323
Hemicellulose	13.7	0.322
Cellulose	22.9	0.686
ADL	6.3	0.296
AIA	0.15	0.085
ADL/NDF	14.6	0.517
ADL/ADF	21.4	0.724
GE (Kcal per Kg)	4219	11.742

Non-fibrous Carbohydrate (NFC) is calculated using the equation of NRC (2001), NFC% = 100-(%NDF+%CP+% FAT+% Ash); NFE = Nitrogen-free Extract; Acid-detergent lignin; AIA = Acid Insoluble Ash; ADL/NDF = Lignification index based on NDF; ADL/ADF = Lignification index based on ADF, SE = Standard Error

Table 2: *In vivo* DM, OM, CP and ME intake, apparent digestibility coefficients of DM, OM and CP of alfalfa hay

	Alfalfa	SE
DMI		
Kg per day	1.64	0.01
g per kg $BW^{0.75}$	81.2	ND
OMI		
g per day	1.5	0.02
g per $BW^{0.75}$	74.2	ND
CPI		
g per day	0.182	0.001
g per $BW^{0.75}$	9	ND
Digestibility coefficient		
DM	65	1.1
OM	66.7	1.3
CP	64	1.1
DOMD[a] (g per kg DM)	613.2	15.5
Predicted ME[b]		
(MJ per kg DM)	9.6	0.17
ME intake (MJ per day)	15.8	0.404

[a] DOMD = Digestible Organic Matter in the Dry Matter, [b] ME value predicted after AFRC (1993), SE = Standard Error, ND = Non–determined

reported by Coblentz *et al.* (1998) and Kamalak *et al.* (2005a). The ash content, which is an index of mineral contents, was 10.33% in alfalfa hay. The ash content of alfalfa hay was consistent with that reported by Kamalak *et al.* (2005a).

***In vivo* determination:** Table 2 shows comparison of DM, OM, CP and ME intakes, apparent digestibility coefficients and digestible DM, OM and CP contents in sheep. DMI, OMI and CPI) in alfalfa hay were 1.64, 1.5 and 0.182 kg day^{-1} and 81.2, 74.2 and 9 g kg^{-1} W$^{0.75}$. DMD, DMI and CPI of HAM hay obtained in this study were consisted with those reported by Vanzant *et al.* (1998) and Martin *et al.* (2000). The DMD, DMI, CPD and CPI contents of HAM hay were lower than those obtained by Phillips *et al.* (2002).

***In situ* degradation characteristics:** The DM, OM and CP degradability of alfalfa hay in rumen are shown in Table 3. The effective degradability of DM, OM and CP of alfalfa hay at the ruminal passage rate of 0.02 h^{-1} was 64, 61.23 and 49.6%, respectively.

The disappearance of DM, OM and CP increased with time of incubation in the rumen (Table 3). These values were in line with those of Komprda *et al.* (1993). They incubated Lucerne (Medicago sativa), harvested at different stages of maturity, for 48 h and showed that the disappearance of OM decreased linearly by up to advancing maturity. They also observed a 22% reduction in CP disappearance with advancing stage of maturity (Komprda *et al.*, 1993). Decreases in degradation could be attributed to an increased lignification process in the cell wall, because lignified tissues limit feed intake and occupy space in the rumen, which may in turn reduce the attachment of bacteria to substrates (Kaya *et al.*, 2004).

Overall, the cumulative disappearance pattern for nutrients appears to decrease linearly with advancing maturity, but slight differences in cumulative disappearance reported in the literature could be due to differences in forage sources, stage of maturity and environmental conditions (Kaya *et al.*, 2004).

In the current study, rapidly degradable fraction and slowly degradable fraction of DM were similar in alfalfa hays (Table 3). These values were in line with those of Seker (2002). The mean values obtained for DM potential degradability in the HAM hay (72.43%) is similar to that obtained by Balde *et al.* (1993) for alfalfa (77.2%). The slight differences between the current study and those of Andrighetto *et al.* (1993) and Kamalak *et al.* (2005a) might be due in part to the different plant species used.

Table 3: *In situ* dry matter, organic matter and protein degradabilities (%) of alfalfa hay

	Dry matter degradability SE	Organic matter degradability SE	Protein degradability SE
0 h	33.4	32.4	21.7
4 h	37	35.4	24.85
8 h	54.6	52.7	27.4
16 h	61	60.4	39.15
24 h	70.46	68.2	50
48 h	71.26	69.25	59.85
72 h	71.4	71.2	60.9
a	34.43	32.4	21.7
b	38	35.1	44.1
c	0.091	0.1	0.038
Effective degradability*	64	61.23	49.6

*Effective degradability = a+bc/ (k+c), a = soluble fraction (%), b = fermentable fraction (%), c = degradation rate of b (% h^{-1}), k = outflow rate per hour, 0.02

Table 4: Organic matter digestibility, gas production (mL) and estimated parameters of Alfalfa hay of different incubation times

Time (h)	2	4	6	8	12	24	48	72	96
	18.4	27	45.02	50.3	54.8	64.4	69.8	72.2	73.1
Estimated parameters									
	a		b	(a+b)		c	OMD		ME
	0.9		68.7	69.6		0.137	71.2		10.96

a = the gas production from the immediately soluble fraction (mL); b = the gas production from the immediately insoluble fraction (mL); c = the gas production rate constant for the insoluble fraction (% h^{-1}); (a+b) = potential gas production (mL); OMD: Organic Matter Digestibility (% of DM); ME: Metabolisable Energy (MJ per kg DM)

Rapidly and slowly degradable fractions of OM were 32.4 and 35.1% in alfalfa hay, respectively. Karsli *et al.* (2002a) found that mean (a), (b) and (c) values of OM were 28.4, 43.3 and 28.3, respectively for alfalfa hay. The values obtained in the correct study for alfalfa hay were also in line with those of Karsli *et al.* (2002a).

Rapidly and slowly degradable fractions of CP observed in the study for alfalfa hay were in range of result reported for alfalfa by Coblentz *et al.* (1998). The difference between the current study and those Michalet-Dorean and Ould-Ban (1992) and Elizalde *et al.* (1999) might be due in chemical compositions, CP extent and harvested time.

Gas production characteristics: Gas production data during the fermentation period are given in Table 2. The cumulative volume of gas production increased with increasing time of incubation. Gas production at 96 h incubation ranged between 73.1 mL per 200 mg of dry matter for alfalfa hay.

The estimated parameters (a, b, a+b and c) of alfalfa hay were 0.9, 68.7, 69.6 % and 0.137 h^{-1}, respectively. The OMD and ME contents of alfalfa hay were 71.2% and 10.96 MJ kg^{-1} DM).

Gas production from the fermentation of forages were measured at 2, 4, 6, 8, 12, 24, 48, 72 and 96 h *in vitro* gas tests adapted to describe the kinetics of fermentation

on the modified exponential model y = a+b[(1-Exp(-ct)] (Ørskov and McDonald, 1979), although there are other models available to describe the kinetics of gas production, the Ørskov and McDonald equation (1979) was chosen because the relationship of its parameters with intake, digestibility and degradation characteristic of forages and concentrate feedstuffs had been documented (Blummel and Orskov, 1993). Cumulative gas production and estimated parameters in ALF hay were comparable to those reported by Kamalak *et al.* (2005a).

The value for a, intercept, in alfalfa hay was 0.9 in this study. The soluble fraction makes it easily attachable by ruminal microorganisms and leads to much gas production (Blummel *et al.*, 1997a).

The gas volumes at asymptote described the fermentation of the insoluble fraction and was 68.7 in alfalfa hay. The gas volumes at asymptote have the advantage for predict feed intake. Blummel and Ørskov (1993) found that gas volume at asymptote could account for 88 % of variance in intake.

Rate of gas production (c) expressed in % h^{-1}, was 0.137 in alfalfa hay. Deaville and Givens (2001) have reported that carbohydrate fraction could be affected to kinetics of gas production.

Potential extent of gas production (a+b) expressed in mL, was 69.6 in alfalfa hay. It is well known that gas production is basically the result of fermentation of carbohydrates to acetate, propionate and butyrate (Getachew *et al.*, 1998), whereas, protein fermentation dose not lead to much gas production (Khazaal *et al.*, 1995). The current finding agrees with *In situ* studies on perennial legumes and grasses (Hoffman *et al.*, 1993). additionally, kinetics of gas production is depended on at relative proportions of soluble, insoluble but degraded and undegradable particles of the feed (Getachew *et al.*, 1998).

OMD and ME content were 71.2% and 10.96 MJ kg^{-1} DM, respectively. The decrease in digestibility is due to increase in concentration of cell wall contents (Wilson *et al.*, 1991), lignin content in mature plant (Morrison, 1980) and decrease in leaf/stem ratio (Hides *et al.*, 1983). Menk *et al.* (1979) suggested that gas volume at 24 h after incubation has been relationship with metabolisable energy in feedstuffs. Sommart *et al.* (2000) reported that gas volume is a good parameter from which to predict digestibility, fermentation end product and microbial protein synthesis of the substrate by rumen microbes in the *in vitro* system. Additionally, *in vitro* dry matter and organic matter digestibility were shown to have high correlation with gas volume (Sommart *et al.*, 2000). Gas volumes also have shown a close relationship with feed intake (Blummel *et al.*, 1997a) and growth rate in cattle (Blummel and Orskov, 1993).

Table 5: Comparison of nutrient contents in alfalfa hay determined by *in vivo*, *In situ* and *in vitro* gas production techniques

Nutrient contents	Method	Alfalfa hay	SE	Sig.
DMI, (g DM $kg^{-1}W^{0.75}$)	*in vivo*	81.2		
	Nylon bag	76		
	Gas production	80.6		
DMD, %	*in vivo*	65		
	Nylon bag-24h DM degradation	70.46		
	48 h DM degradation	71.26		
	72 h DM degradation	71.4		
	ED of DM	64		
	Gas production	76.02		
OMD, %	*in vivo*	66.7		
	Nylon bag-24 h OM degradation	68.2		
	48 h OM degradation	69.25		
	72 h OM degradation	71.2		
	ED of OM	61.23		
	Gas production	71.2		
CPD, %	*in vivo*	64		
	Nylon bag-24 h CP degradation	50		
	48 h CP degradation	59.85		
	72 h CP degradation	60.9		
	ED of CP	49.6		
ME, MJ kg^{-1} DM	*in vivo*	9.6		
	Gas production	10.96		

Comparison of the nutrient content obtained by *in vivo* method and laboratory (*in vitro*) method (Table 5) indicated that nylon bag technique was a good method to estimate *in vivo* DM and CP digestibility. The nylon bag and gas production techniques were similar method to estimate *in vivo* OM digestibility.

CONCLUSION

Calculation of digestibilities using the *In situ* technique values similar to actual digestibilities in alfalfa hay. However, in the gas production, the values of ME in alfalfa hay were close to the value obtained from the *in vivo* study.

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