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Botanical traits, protein and carbohydrate fractions, ruminal degradability and energy contents of alfalfa hay harvested at three stages of maturity and in the afternoon and morning

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ABSTRACT

Little information is available about nutrient profiles and availability of alfalfa harvested at different stages of maturity and harvested at different times during the day in relation to diet formulation for dairy cows. The objective of this study was to investigate the effect of stage of maturity and cutting time of alfalfa hay on botanical traits, nutrient profiles and *in situ* degradability of protein and carbohydrates and calculated energy content. Alfalfa was cut at early bud (June 15/16), late bud (June 26/27) and early flower stage (July 18/19) both in the afternoon (06:00 pm) and the next morning (06:00 am). With advancing maturity of alfalfa, leaf content, leaf:stem ratio, calculated energy values, crude protein (CP), *in situ* digestibility's (especially at 12 and 36 h of incubation) and nitrogen (N) to energy [organic matter (OM), carbohydrates (CHO)] ratios decreased ($P<0.05$). While, neutral detergent fiber, acid detergent fiber, fiber associated CP (NDICP) and total CHO increased ($P<0.05$) with advancing maturity of alfalfa. Protein and CHO fractions (defined according to Cornell net carbohydrate and protein system) associated with different degradation characteristics stayed consisted with advancing maturity, except for intermediate degradable protein (PB2), which decreased at the early flower stage compared with early and late bud stages ($P=0.03$). Alfalfa harvested in the afternoon tended to have a higher leaf portion and leaf:stem ratio ($P=0.06$) and contained 13 g/kg CHO more soluble carbohydrates (TESC, *i.e.* CA; $P<0.01$), 27 g/kg CP more PB2 ($P=0.02$), and 0.33, 0.33, 0.27 MJ/kg DM more ($P<0.05$) net energy for maintenance, gain and lactation production, respectively, and had an improved

Abbreviations: ADF, acid detergent fiber; ADICP, acid detergent insoluble crude protein; aNDF, neutral detergent fiber; CA, soluble carbohydrate; CB1, rapidly degradable carbohydrate; CB2, intermediate degradable carbohydrate; CB3, slowly degradable carbohydrate; CC, undegradable carbohydrate; CHO, total carbohydrates; CNCPS, Cornell net carbohydrate and protein system; CP, crude protein; CT, cutting time; DM, dry matter; EE, ether extract; NDICP, neutral detergent insoluble crude protein; NE_g, net energy for gain; NE_{lp}, net energy lactation at production level of intake; NE_m, net energy for maintenance; NFC, none fiber carbohydrates; NRC, national research council; OM, organic matter; PA, solublizable protein; PB1, rapidly degradable protein; PB2, intermediate degradable protein; PB3, slowly degradable protein; PC, undegradable protein; SM, stage of maturity; TESC, total ethanol soluble carbohydrate.

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rapidly degradable N to CHO ratio (PA:CA; 66 *vs.* 76 g/kg; P=0.02) compared with alfalfa harvested in the morning. Cutting time had no impact on *in situ* degradability of alfalfa hay. In conclusion, nutrient availability of alfalfa hay, grown under semi arid climate condition, was not only influenced by stage of maturity but also by cutting time. In general, alfalfa harvested at early and late bud but in the afternoon had the highest nutrient levels for dairy production.

1. Introduction

Cultivated alfalfa (*Medicago sativa* L.) is one of the major forage crops in the world (Hanson et al., 1988) and the most important forage crop for dairy rations in Iran (Kowsar et al., 2008). Alfalfa contains high nutrients levels, high digestibility, and unique proportion of structural to non-structural components (Yu et al., 2003). Botanical traits, nutritive value and crude protein (CP) and carbohydrate (CHO) fractions of alfalfa are influenced by cultivar, stage of maturity (SM) (Elizalde et al., 1999; Yu et al., 2003; Coblentz et al., 2008), climate condition (Weir et al., 1960; Lamb et al., 2003) and cutting time (CT) due to accumulation of non-structural CHO during the day (Burns et al., 2007; Brito et al., 2008, 2009).

Various CP and CHO fractions present in feed differ in rate and extent of ruminal degradation. These fractions influence the amount of CP and CHO degraded in the rumen and escaping to the lower digestive tract (Lanzas et al., 2007a,b; Jonker et al., 2011). Furthermore, the knowledge of these CP and CHO fractions and degradation is used in modern diet formulation programs such as Cornell net carbohydrate and protein system (CNCPS; Lanzas et al., 2007a,b) and National Research Council (NRC, 2001) to formulate ruminant diets. Therefore, information about CP and CHO fractions and degradability (NRC, 2001; Lanzas et al., 2007a,b) and predicted energy values (NRC, 2001) should be taken in to consideration when formulating diets for ruminants. However, information about the effect of alfalfa CT at different stages of maturity on these nutritional properties is lacking. Our objectives were to investigate botanical traits, protein and carbohydrate fractions, *in situ* ruminal degradability and energy content of alfalfa harvested at three stages of maturity in the afternoon and next morning.

2. Materials and methods

2.1. Alfalfa plots management

A second year alfalfa field (20 m×24 m) seeded with *cv.* Ranger at the Research Farm of Ferdowsi University of Mashhad (Mashhad, Iran; 36°17′52.8″N, 59°36′20.52″E) was used in this study. The whole field was harvested before the experiment at April 6, 2010 and irrigated every 10 days during experiment. The first cut at May 11, 26 and 30 for early bud, late bud and early flower, respectively was not used for this study.

Six plots (4 m×4 m each) within 5 replicate blocks were randomly assigned to 6 treatments in a factorial arrangement (3 SM×2 CT). The three SM were early bud, late bud and early flower and two CT were at 06:00 pm and the next morning at 06:00 am. The SM was determined according to Kalu and Fick (1981). Briefly, a quadrate (250 cm^2) was randomly thrown in each plot (one time) and all stems above 3 cm stubble height inside the quadrate (*ca.* 70–80 stems) were used to calculate the mean SM for each plot. In total, there were 10 plots for each SM from which half was cut at 06:00 pm and the other half at 06:00 am, when alfalfa reached the appropriate SM (Table 1). At each harvest, an area of 3 m×3 m was manually clipped using a small scythe at *ca.* 5 cm above the soil surface.

Immediately after cutting, twenty stems were randomly selected from each plot to separate leafs and stems by hand. Alfalfa leaf, stem, and whole plant dry matter (DM) content were determined by oven drying for 48 h at 60 °C. Remaining fresh alfalfa harvested from each plot was air dried in the shade (*ca.* 10–15 days). After air drying, alfalfa hay samples were chopped using a hay chopper with 20 mm screen (Agri-Equip, Nasr Co., Isfahan, Iran). The hay from the first, second and third blocks were pooled to one sample and hay from the fourth and fifth blocks were pooled to another sample to generate sufficient material for chemical analysis and *in situ* degradability measurements.

Table 1
Date and climate condition in Mashhad, Iran during alfalfa cutting in 2010.

Date of cutting	Min[a]	Max	GDD	TTC	Sunrise	Sunset
Early bud, 06:00 pm (June 15)	18	35	22	28	05:14	19:51
Early bud, 06:00 am (June 16)	18	35	22	19	05:14	19:51
Late bud, 06:00 pm (June 26)	19	36	23	27	05:16	19:54
Late bud, 06:00 am (June 27)	19	37	23	20	05:17	19:54
Early flower, 06:00 pm (July 18)	24	36	25	27	05:28	19:48
Early flower, 06:00 am (July 19)	23	36	24	24	05:29	19:48

[a] Min: minimum temperature (°C); Max: maximum temperature (°C); GDD: growing degree day was calculated daily by subtracting 5 °C from the average of the maximum and minimum temperatures for that day (Coblentz et al., 2008); TTC: temperature at time of cutting; the time of sunset and sunrise at that day; there was no rainfall at cutting dates; data for climate condition were collected from weather station located close to the experimental field (Mashhad Meteorological Network Station, Mashhad, Iran).

2.2. Chemical analysis

Chopped alfalfa was ground through a 2-mm screen (Laboratory Hammer Mill, Christry & Norris Ltd., England) before *in situ* incubations and ground through a 1-mm screen (Retsch ZM-1, Brinkmann Instruments Ltd., ON, Canada) for chemical analysis. Standard procedures described by the Association of Official Analytical Chemists (AOAC, 1990) were used to determine dry matter (DM; AOAC 930.15), ash (AOAC 942.05), crude protein (CP; AOAC 984.13) and ether extract (EE; AOAC 954.02). Neutral detergent fiber assayed with heat stable alpha amylase (aNDF) and acid detergent fiber (ADF) was determined with the ANKOM A200 Filter Bag technique (Ankom Technology, Fairport, NY, USA) according to Van Soest et al. (1991). Neutral detergent insoluble CP (NDICP) and acid detergent insoluble CP (ADICP) were determined by Kjeldahl-N analysis of the aNDF and ADF bag residues, respectively, as described by Licitra et al. (1996). The reported ADF and aNDF were corrected for NDICP and ADICP, respectively, but not for ash. Lignin (sa) was determined by soaking the ADF filter bag residue in sulfuric acid for 3 h followed by nine washes with water (AOAC 973.18). Starch was measured using the Megazyme total starch assay kit (Megazyme International Ltd., Wicklow, Ireland; McCleary et al., 1997). Total ethanol soluble carbohydrates (TESC) were determined according procedures described by Hall et al. (1999). Non-protein N (NPN) not precipitated by trchloroacetic acid and sodium bicarbonate/phosphate buffer soluble crude protein (SCP) were determined according to Licitra et al. (1996). Chemical analysis was performed in duplicate. Non-fiber carbohydrates [NFC = 1000 − (aNDF + CP + EE + ash)] and total carbohydrates [CHO = 1000 − (CP + EE + ash)] were calculated according to NRC dairy (2001).

2.3. Protein and carbohydrate fractionation

The CNCPS was used to divide CP and CHO into five fractions each (Sniffen et al., 1992; Lanzas et al., 2007a,b). The CP content was divided into instantaneously solubilizable protein A (PA; *i.e.* NPN), completely undegradable CP (PC; *i.e.* ADICP) and potentially degradable true protein (PB; *i.e.* CP–NPN–ADICP). The PB was further sub-divided into rapidly (PB1; *i.e.* SCP–NPN), intermediately (PB2; *i.e.* PB–PB1–PB3), and slowly (PB3; *i.e.* NDICP–ADICP) degradable true protein. Normally, the revised CNCPS scheme fractionates CHO into eight fractions (Lanzas et al., 2007a). However, alfalfa hay does not contain volatile fatty acids (CA1) and lactic acid (CA2) and only small amounts of other organic acids (CA3) (Hall et al., 1999; Lanzas et al., 2007a; Jonker et al., 2010). Therefore, we divided CHO into five fractions; instantaneously solublizable CHO (CA; *i.e.* TESC), rapidly degradable CHO (CB1; *i.e.* starch), intermediately degradable CHO (CB2; *i.e.* NFC–TESC–starch), slowly degradable CHO (CB3; *i.e.* aNDF − CC), and undegradable CHO (CC; *i.e.* aNDF×(lignin/aNDF)×2.4), composed of completely undegradable NDF (Lanzas et al., 2007a).

2.4. Calculated energy contents

Total digestible nutrient was calculated using *in situ* aNDF digestibility at 36 h and total digestible CP, fatty acid, and NFC calculated using the summative approach described in NRC dairy (2001). Net energy lactation at production level of intake (NE_{lp}) was calculated according to NRC dairy (2001) and net energy for maintenance (NE_m) and growth (NE_g) were calculated according to NRC beef (1996).

2.5. Rumen incubation procedure

For *in situ* rumen incubation, three-rumen fistulated, non-pregnant, dry Holstein Frisian dairy cows were used, which had been reviewed and approved by the Animal Care Committee of the University of Saskatchewan (Animal use protocol # 19910012). Cows were individually housed in pens at the experimental farm of the University of Saskatchewan (Saskatoon, SK, Canada) and were cared for according to the Canadian Council on Animal Care guidelines (1993). The cows had free access to water and were fed 15 kg DM/day total mixed ration twice daily in equal portions at 08:00 am and 04:00 pm. The total mixed ration consisted ing/kgDM of 550 barley silage, 125 alfalfa hay, 50 dehydrated alfalfa and 275 concentrates. Ruminal degradability of DM, organic matter (OM), CP and aNDF was determined at 12, 36 and 72 h of incubation by the 'all out method' (Yu et al., 2004). Number of bags, amount of sample per bag surface area and washing procedure after withdrawal from rumen were recently described in detail by Jonker et al. (2011). Rumen incubation was carried out in one run and the two pooled blocks were used as replicate for the *in situ* trial. Incubation residues from the treatment bags were combined within time per block.

2.6. Statistical analysis

Experimental plots ($n = 30$; 5 blocks×6 plots) were established as randomized complete block design with factorial arrangement of two main factors (3 SM×2 CT). Data from five original blocks were used to test the effects of SM and CT and their interaction on botanical traits, while chopped alfalfa hay samples from two pooled blocks were used to test the effect on chemical composition, protein and carbohydrate fractions, *in situ* degradability and energy content. Data were

Table 2
Botanical traits and detailed chemical components for alfalfa hay cut at three stages of maturity and in the afternoon and next morning.

Items[b]	Stage of maturity (SM)			SEM	Cutting time (CT)		SEM	Level of significance[a]	
	Early bud	Late bud	Early flower		06:00 am	06:00 pm		SM	CT
Botanical traits									
DM (g/kg)	210[b]	278[a]	317[a]	15.2	263	276	13.0	<0.01	0.45
Leaf content (g/kg DM)	354[a]	292[b]	269[b]	10.0	293	316	8.2	<0.01	0.06
Leaf:stem ratio	0.55[a]	0.42[b]	0.37[b]	0.02	0.42	0.47	0.02	<0.01	0.06
Detailed chemical components									
ADF (g/kg DM)	355[b]	380[b]	430[a]	12.4	394	382	10.1	0.01	0.43
ADICP (g/kg CP)	58	72	77	6.6	71	67	5.5	0.13	0.55
aNDF (g/kg DM)	425[b]	443[b]	491[a]	14.0	456	449	11.4	0.03	0.67
Ash (g/kg DM)	107[a]	102[a]	81[b]	2.6	99	95	2.1	<0.01	0.18
CHO (g/kg DM)	645[c]	678[b]	734[a]	7.0	685	689	5.7	<0.01	0.59
CP (g/kg DM)	220[a]	195[b]	162[c]	4.8	192	192	3.9	<0.01	0.96
EE (g/kg DM)	24	25	23	1.0	24	24	0.8	0.65	0.86
Lignin (sa; g/kg DM)	80	84	92	3.3	87	84	2.7	0.08	0.49
N:CHO (g/kg)	54[a]	46[b]	35[c]	1.6	45	45	1.3	<0.01	0.86
NDICP (g/kg CP)	128[b]	114[b]	145[a]	5.1	130	128	4.2	<0.01	0.77
N:OM (g/kg)	39[a]	35[b]	28[c]	0.9	34	34	0.7	<0.01	0.84
NFC (g/kg DM)	225	235	243	8.5	228	240	7.0	0.37	0.28
NPN (g/kg SCP)	799	800	771	33.0	803	777	30.1	0.63	0.35
SCP (g/kg CP)	572	581	585	12.4	592	567	11.1	0.59	0.054
Starch (g/kg DM)	7	9	8	0.8	8	8	0.6	0.24	0.67
TESC (g/kg DM)	61	66	63	1.9	59[y]	68[x]	1.5	0.23	<0.01
TP (g/kg CP)	543	535	548	25.5	524	560	24.4	0.78	0.051

[a]There was no interaction between SM and CT; SEM, standard error of means; means with different letters (a, b and c for SM) and (x and y for CT) within the same row differ (P<0.05).
[b]DM, dry matter content of fresh alfalfa; ADF, acid detergent fiber; ADICP, acid detergent insoluble crude protein; aNDF, neutral detergent fiber; CHO, total carbohydrate calculated as 1000 – (CP + EE + ash) (NRC, 2001); N:CHO, ratio between nitrogen and total CHO; NDICP, neutral detergent insoluble crude protein; NFC, non-fiber carbohydrates calculated as 1000 – (CP + EE + Ash + aNDF) (NRC, 2001); N:OM, ratio between nitrogen and organic matter; NPN, non-protein nitrogen; SCP, buffer soluble protein; TESC, total ethanol soluble carbohydrates; TP, true protein.

analyzed as randomized complete block design using proc mixed of SAS 9.2 (2003). The statistical model used in SAS 9.2 (2003) was as following:

$$Y_{ijk} = \mu + B_k + \mathrm{CT}_i + \mathrm{SM}_j + \mathrm{CT}_i \times \mathrm{SM}_j + e_{ijk}$$

where Y_{ijk} is the observation of the dependent variable *ijk*; μ is the fixed effect of population mean for the variable; B_k is the random effect of block ($k = 5$ for agronomic traits and $k = 2$ for the other measurements); CT_i is the fixed effect of cutting time ($i = 2$; 06:00 am and 06:00 pm); SM_j is the fixed effect of stage of maturity ($j = 3$; early bud, late bud and early flower); $\mathrm{CT}_i \times \mathrm{SM}_j$ is the fixed effect of interaction between factor CT at level *i* and the factor SM at level *j* and e_{ijk} is the random error associated with the observation *ijk*. The effect of $\mathrm{CT}_i \times \mathrm{SM}_j$ was not significant and was therefore excluded from the model. The Fisher's protected least significant difference (LSD) test was used for multiple treatment comparisons using the LSMEAN statement of SAS 9.2 (SAS, 2003) with letter groupings obtained using the SAS pdmix800 macro (Saxton, 1998). For the different statistical tests, significance was declared at $P \leq 0.05$ and trends at $P \leq 0.10$, unless otherwise stated.

3. Results

3.1. Botanical traits

Alfalfa at early bud had a lower DM content compared with alfalfa at late bud and early flower (P<0.05) and DM was similar between both CT (Table 2). Alfalfa at early bud had higher (P<0.05) leaf:stem ratio and leaf content compared with alfalfa at late bud and early flower and cutting alfalfa in the afternoon tended to increase the leaf:stem ratio by 0.05 (P=0.06) and the leaf content by 23 g/kg DM (P=0.06; Table 2) compared with cutting alfalfa in the morning.

3.2. Chemical composition

The EE, NFC, ADICP, TESC, starch, SCP, true protein (TP) and NPN were similar among stages of maturity (Table 2). Alfalfa at early flower had higher (P<0.05) ADF, aNDF, and NDICP and lower (P<0.05) ash, compared with alfalfa at early and late bud. The CP, N:CHO, N:OM decreased with advancing SM (P<0.05) while CHO increased (P<0.05) and ligning (sa) tended to increase (P<0.10) with advancing SM. Alfalfa cut in the afternoon had a higher TESC content (P<0.05)and tended to have a higher TP (P=0.051) and lower SCP content (P=0.054; Table 2) compared with the morning cut; other chemical components were similar between cutting times.

Table 3
Crude protein and carbohydrate fractions in alfalfa hay cut at three stages of maturity and in the afternoon and next morning.

Items[b]	Stage of maturity (SM)			SEM	Cutting time (CT)		SEM	Level of significance[a]	
	Early bud	Late bud	Early flower		06:00 am	06:00 pm		SM	CT
Carbohydrate fractions (g/kg CHO)									
CA	94	98	86	3.0	86[y]	99[x]	2.4	0.06	<0.01
CB1	10	13	11	1.2	11	12	0.9	0.35	0.72
CB2	242	234	235	12.2	237	239	9.9	0.92	0.87
CB3	358	356	366	11.5	362	358	9.4	0.83	0.76
CC	295	297	302	9.1	304	292	7.5	0.85	0.32
Protein fractions (g/kg CP)									
PA	457	465	452	25.5	476	440	2.4	0.78	0.051
PB	486	462	470	29.9	453	493	28.7	0.57	0.055
PB1	115	116	133	17.7	116	127	15.7	0.60	0.55
PB2	301[a]	304[a]	269[b]	14.3	278[y]	305[x]	13.6	0.03	0.02
PB3	70	42	68	8.0	59	61	6.5	0.07	0.77
PC	58	72	77	6.4	71	67	5.5	0.13	0.55
Nitrogen to carbohydrate ratios (g/kg)									
PA:CA	83[a]	73[b]	56[c]	5.8	76[x]	66[y]	5.5	<0.01	0.02
PB:CB	44[a]	36[b]	28[c]	2.1	34	37	1.9	<0.01	0.14
PC:CC	11	11	9	1.4	10	10	1.1	0.48	0.98

[a]There was no interaction between SM and CT; SEM, standard error of means; means with different letters (a, b and c for SM) and (x and y for CT) within the same row differ (P<0.05).
[b]Carbohydrate fractions according to CNCPS (Lanzas et al., 2007a) include: CA, simple sugars measured as total ethanol soluble CHO; CB1, starch; CB2, soluble fiber calculated as CHO − (CA + CB1 + CB3 + CC); CB3, degradable NDF calculated as aNDF − CC; CC, undegradeble NDF calculated as (NDF×(lignin (sa)/NDF)×2.4)/CHO×100; protein fractions according to CNCPS (Lanzas et al., 2007b) include PA, fraction of CP that is instantaneously solubilized at time zero and determined as NPN; PB1, soluble true protein calculated as buffer soluble CP minus non-protein nitrogen; PB2, intermediate degradable true protein calculated as CP − (PA + PB1 + PB3 + PC); PB3, slowly degradable true protein calculated as NDICP − ADICP; PC, undegradable CP determined as ADICP.

3.3. Protein and carbohydrate fractions and nitrogen to carbohydrate ratios

All CHO and CP fractions were similar among the three SM except for PB2, which was lower (P<0.05) at early flower compared with early and late bud (Table 3). The CA tended to decrease with advancing maturity (P=0.06) and PB3 tended to be lower in alfalfa at late bud compared with the other two stages (P=0.07). Alfalfa cut in the afternoon had a higher CA and PB2 (P<0.05) and tended to have a lower PA and higher PB (P<0.10) compared with alfalfa cut in the morning (Table 3).

The ratio of PA:CA and the PB:CB decreased with advancing SM (P<0.01), while PC:CC ratio was similar among three SM (Table 3). Alfalfa cut in the afternoon had a lower PA:CA ratio (P=0.02), but similar PB:CB and PC:CC ratio compared with alfalfa cut in the morning.

3.4. In situ nutrient degradability

The *in situ* degradable DM (DDM) at 12 and 36 h (g/kg DM), DCP at 12, 36 and 72 h (g/kg DM; Table 4), DOM at 12 h (g/kg OM), DCP at 12 and 36 h (g/kg CP) and DNDF at 12 h (g/kg aNDF; Table 5) decreased with advancing SM (P<0.05). However, SM did not affect extend of total tract degradability for any parameter measured (at 72 h incubation), except for DCP (g/kg DM). Cutting time had no impact on *in situ* degradability parameters measured at any incubation time (Tables 4 and 5).

3.5. Calculated energy contents

The NE_{lp}, NE_m and NE_g were higher in alfalfa at early bud than late bud and early flower (P=0.03; Fig. 1) and showed an decreasing trend with advancing SM. Alfalfa cut in the afternoon had a higher NE_m, NE_{lp} and NE_g compared with alfalfa cut in the morning (Fig. 1).

4. Discussion

4.1. Effect of stage of maturity

Results from our study indicate that alfalfa SM affects its botanical composition (see Table 2) in agreement with published results (Thompson et al., 2000; Sheaffer et al., 2000; Lamb et al., 2003). Alfalfa leaves have a higher CP, higher digestibility and lower fiber content compared with alfalfa stems (Van Soest, 1994). Therefore, the decreased leaf:stem ratio with advancing SM found in this study likely caused reduced CP, reduced *in situ* degradability and increased structural CHO (*e.g.* aNDF and ADF) similar to published results (Llamas-Lamas and Combs, 1990; Balde et al., 1993; Yu et al., 2003, 2004).

Table 4
In situ degradability of dry matter (DM), organic matter (OM), crude protein (CP) and neutral detergent fiber (NDF) after 12, 36 and 72 h of ruminal incubation for alfalfa hay cut at three stages of maturity and in the afternoon and next morning.

Items[b]	Stage of maturity (SM)			SEM	Cutting time (CT)		SEM	Level of significance[a]	
	Early bud	Late bud	Early flower		06:00 am	06:00 pm		SM	CT
Degradable DM (g/kg DM) after 12, 36 and 72 h of ruminal incubation									
DDM_{12}	437[a]	409[a,b]	370[b]	18.4	404	407	17.1	0.02	0.87
DDM_{36}	571[a]	550[a,b]	516[b]	11.6	542	549	9.51	0.03	0.59
DDM_{72}	614	598	549	16.0	590	584	13.1	0.06	0.78
Degradable OM (g/kg DM) after 12, 36 and 72 h of ruminal incubation									
DOM_{12}	418	393	374	16.4	391	399	14.9	0.09	0.55
DOM_{36}	551	529	519	10.5	526	540	8.6	0.16	0.28
DOM_{72}	592	580	553	14.3	575	575	11.6	0.21	0.99
Degradable CP (g/kg DM) after 12, 36 and 72 h of ruminal incubation									
DCP_{12}	134[a]	115[b]	85[c]	4.7	111	111	4.3	<0.01	0.95
DCP_{36}	161[a]	140[b]	103[c]	5.6	135	135	4.6	<0.01	0.97
DCP_{72}	172[a]	149[a]	113[b]	7.5	146	143	6.1	<0.01	0.78
Degradable aNDF (g/kg DM) after 12, 36 and 72 h of ruminal incubation									
$DNDF_{12}$	76	69	63	3.7	70	69	3.0	0.12	0.85
$DNDF_{36}$	163	151	172	9.2	158	167	7.5	0.32	0.42
$DNDF_{72}$	178	180	180	7.2	184	175	5.9	0.97	0.28

[a]There was no interaction between SM and CT; SEM, standard error of means; means with different letters (a, b and c for SM) and (x and y for CT) within the same row differ (P<0.05).
[b]The aNDF was assayed with heat stable alpha amylase and sodium sulfite and expressed inclusive residual ash.

The only protein fraction that differed with SM was PB2 (Table 3) and this fraction was the largest PB fraction similar to findings of Sniffen et al. (1992) and Elizalde et al. (1999), while PB3 was the largest PB fraction in the study of Yu et al. (2003). This fraction was higher at the most mature early flower stage, which was opposite to findings of Yu et al. (2003). The sum of PA and PB is considered as total tract degradable CP by CNCPS (Sniffen et al., 1992). The sum of these two fractions was higher than *in situ* degradable protein content at 72 h (see Table 3). This implies that infinite protein degradation was not reached by 72 h of *in situ* incubation or PA + PB fractions might not be a reliable predictor for total tract degradable protein content of alfalfa as stated previously by Haugen et al. (2006) and Jonker et al. (2011).

Reduction in *in situ* DCP content (g/kg DM; see Table 4) with advancing SM was primarily due to reduction in CP concentration with advancing SM. Reduction in DCP concentration (g/kg CP) at 12 and 36 h of ruminal incubation for early flowering alfalfa was likely caused by higher cell wall associated protein (NDICP) which is harder to access for ruminal microbes (Sniffen et al., 1992). However, the magnitude of differences among stages of maturity for DCP reduced with advancing ruminal incubation time, which suggests that site of protein degradation was more affected by SM than total tract extend of protein degradation.

Table 5
In situ degradability of organic matter (OM), crude protein (CP) and neutral detergent insoluble fiber (NDF) after 12, 36 and 72 h of ruminal incubation for alfalfa hay cut at three stages of maturity and in the afternoon and next morning.

Items[b]	Stage of maturity (SM)			SEM	Cutting time (CT)		SEM	Level of significance[a]	
	Early bud	Late bud	Early flower		06:00 am	06:00 pm		SM	CT
Degradable OM (g/kg OM) after 12, 36 and 72 h of ruminal incubation									
DOM_{12}	468[a]	438[a,b]	407[b]	18.1	434	441	16.4	0.04	0.63
DOM_{36}	617	590	565	12.8	584	597	10.5	0.07	0.41
DOM_{72}	662	646	602	16.0	638	635	13.1	0.08	0.87
Degradable CP (g/kg CP) after 12, 36 and 72 h of ruminal incubation									
DCP_{12}	608[a]	588[a]	524[b]	23.4	571	576	21.9	0.01	0.77
DCP_{36}	734[a]	718[a]	639[b]	15.1	696	697	12.3	<0.01	0.97
DCP_{72}	783	763	696	24.5	754	740	20.7	0.07	0.63
Degradable NDF (g/kg aNDF) after 12, 36 and 72 h of ruminal incubation									
$DNDF_{12}$	178[a]	142[b]	140[b]	4.7	156	151	3.8	<0.01	0.35
$DNDF_{36}$	385	341	351	13.9	347	371	11.4	0.14	0.18
$DNDF_{72}$	420	406	368	16.0	405	391	13.1	0.12	0.47

[a]There was no interaction between SM and CT; SEM, standard error of means; Means with different letters (a, b and c for SM) and (x and y for CT) within the same row differ (P<0.05).
[b]The NDF was assayed with heat stable alpha amylase and sodium sulfite and expressed inclusive residual ash.

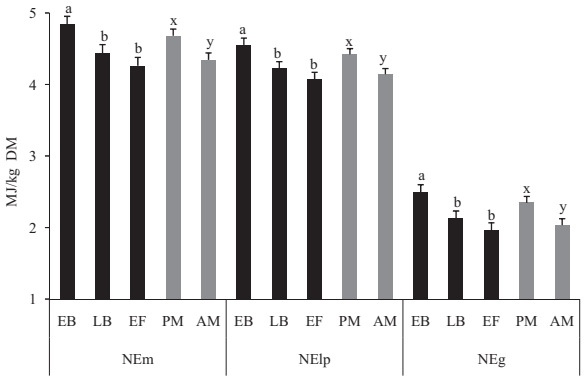

Fig. 1. Calculated energy contents (NRC basis) of alfalfa hay cut at three stages of maturity (SM; EB: early bud, LB: late bud and EF: early flower) and in the afternoon (06:00 pm) and next morning (06:00 am); net energy for lactation at production level of intake (NE_{lp}) from NRC dairy (2001); net energy for growth (NE_g) and net energy for maintenance (NE_m) from NRC beef (1996); Level of significance for the effect of SM and cutting time (CT) were P = 0.03 and P = 0.04, respectively. There was no interaction between SM and CT. Bars with different letters (a,b and c for SM; x and y for CT) within the same component differ (P < 0.05).

The CA fraction tended to decrease with advancing SM (Table 3), which was in agreement with the result of Yu et al. (2003). This might be due to reduced leaf:stem ratio with consequent increased cell wall CHO component and declined cellular CHO (Van Soest, 1994). The mean values of CHO fractions were comparable with those reported by Yu et al. (2003) and Lanzas et al. (2007a). Reported concentrations of CB2 and CB3 in alfalfa harvested at vegetative stage were ~420 and ~200 g/kg CHO, respectively (Jonker et al., 2010) which indicates that immature alfalfa contains more soluble fiber and less slowly degradable CHO compared with alfalfa in this study at more advanced SM. Changes in CHO fractions with advancing SM were small which is in agreement with the results of Elizalde et al. (1999) for fresh alfalfa and Yu et al. (2003) for alfalfa hay.

4.2. Effect of cutting time

There was a trend toward higher leaf:stem ratio for alfalfa hay cut in the afternoon (Table 2) which is in agreement with finding of Lechtenberg et al. (1971). This might result from accumulation of photosynthesis end-products during the day in leaves (Lechtenberg et al., 1971; Van Soest, 1994). During the day, PA tended to decrease and PB tended to increase, which is in agreement with Huntington and Burns (2007). During the day, alfalfa leaves did likely use of photosynthesis end-products to convert PA components into true protein (PB; Van Soest, 1994). The higher alfalfa leaf:stem ratio in the afternoon did not result in higher *in situ* degradability compared with alfalfa cut in the morning.

The CA fraction was higher in the afternoon cut compared with the morning cut (Table 3), likely because of accumulation photosynthesis products, which is in agreement with previous published results (Fisher et al., 2002; Burns et al., 2007; Brito et al., 2008, 2009). While these authors found that starch (*i.e.* CB1) and NDF differed between morning and afternoon harvests, these parameters were similar at both cutting times in our study. Factors such as time of cutting, day length, temperature, sunshine intensity and mode of forage conversation can influence CHO fractionation during day (Van Soest, 1994; Burns et al., 2007; Brito et al., 2008, 2009) and might therefore be the cause of differences among studies.

4.3. Nitrogen to energy ratios and energy content

Optimum N:CHO and N:OM ratio for microbial growth in the rumen was reported to be ~32 g N/kg CHO (Sinclair et al., 1991) and ~25 g N/kg OM (Czerkawski, 1986), respectively. In agreement with the results reported by Yu et al. (2004) and Jonker et al. (2011), current findings revealed that alfalfa had excessive N per unit of CHO and OM (see Table 3). Not only should the ratio between available N and CHO be optimum, but the ratio should be synchronized as well to achieve efficient microbial growth and minimize N losses from the rumen (Tamminga et al., 1990; Yu et al., 2004; Jonker et al., 2010, 2011). The synchronization of the N:CHO ratio can be expressed in part by PA:CA and PB:CB ratios. The synchronization between N and CHO improved with advancing SM and PA:CA ratio improved when cutting alfalfa in the afternoon.

The NRC (2001) suggests using *in vitro* NDF degradability at 48 h of incubation to determine total digestible NDF for the calculation of energy values. This duration seems long for evaluation of forage energy content for high producing dairy cows (Oba and Allen, 2005). Therefore, we used a combination of *in situ* $DNDF_{36}$ and summative equation (NRC, 2001) to calculate energy values. In agreement with findings of Weir et al. (1960) and Yu et al. (2003), energy content of alfalfa hay decreased with advancing SM (see Fig. 1). Because leaves are more digestible compared with stems (Van Soest, 1994), the higher energy

content in alfalfa at early bud stage and cut in the afternoon is likely the result of a higher leaf:stem ratio. Energy values reported for this study were lower than previously reported (NRC, 2001; Yu et al., 2003). These lower energy values might have resulted from differences in alfalfa cultivar and environmental factors; in declining order of importance, temperature, light and day length, latitude, water level, fertilization and soil type (Van Soest, 1994).

5. Conclusions

Potential nutrient supply to the animal reduced with advancing alfalfa maturity. With advancing maturity, leaf:stem ratio decreased and consequently, protein content and digestibility decreased, while fiber fractions increased and nitrogen to energy synchronization improved. Cutting alfalfa in the afternoon increased leaf content, soluble carbohydrates concentration, energy values, and improved rapidly degradable nitrogen to carbohydrate synchronization compared with cutting alfalfa in the morning.

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