Determination of nutritive value of wild narrow-leaved clover (*Trifolium angustifolium*) hay harvested at three maturity stages using chemical composition and *in vitro* gas production

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Abstract

The aim of this study was to determine the potential nutritive value of a wild narrow-leaved clover (*Trifolium angustifolium*) hay harvested at 3 maturity stages (pre-flowering, flowering and seeding) in terms of chemical composition and *in vitro* gas production. *In vitro* gas production of *Trifolium angustifolium* hay was determined at incubation times of 0, 3, 6, 12, 24, 48, 72 and 96 h and the gas production kinetics were described using the equation $y = a + b\ (1\text{-}e^{-ct})$. Maturity had a significant effect on both chemical composition and *in vitro* gas production kinetics. Neutral detergent fibre (NDF), acid detergent fibre and condensed tannin (CT) concentrations increased with maturity, while crude protein (CP) concentration decreased from 16.9% pre-flowering to 13.2% at seeding. The condensed tannin concentration of *Trifolium angustifolium* hay ranged from 2.16 to 3.71%.

Estimated gas production decreased with increasing maturity of *Trifolium angustifolium* hay. ME, OMD, a+b, a and CP concentration were negatively correlated with NDF, ADF and CT. It appears that wild *Trifolium angustifolium*, harvested even at the seeding stage, offers considerable potential as medium quality forage for ruminant animals during the winter period.

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Introduction

Forage has an important role in ruminant nutrition in terms of providing energy, protein and minerals, as well as fibre for chewing and rumination. In Mediterranean areas pastures represent the most important forage resource. Narrow-leaved clover (*Trifolium angustifolium*) is a self-reseeding annual legume and has an important role in native pasture in Mediterranean environments. Driouech *et al*. (2008) showed that *Trifolium angustifolium* fixed 131.7 and 146.7 kg/ha/year of N and produced 8.7 and 7.7 t/ha dry matter (DM) in 2006 and 2007, respectively.

Although the nutritive value of many forages at different stages of maturity is well established (Buxton 1996; Kamalak *et al*. 2005a, 2005b; Aydin *et al*. 2007), there is limited information of this nature for narrow-leaved clover (*Trifolium angustifolium*). Accurate prediction of forage quality during the growth cycle would allow targeting of harvest or grazing to optimal levels to meet specific animal requirements (Valente *et al*. 2000). Chemical composition, in combination with *in vitro* gas production, organic matter digestibility (OMD) and metabolisable energy (ME) concentration are widely used to determine the potential nutritive value of forages (Evitayani *et al*. 2004; Fujihara *et al*. 2005; Kamalak *et al*. 2005a, 2005b; Karabulut *et al*. 2007; Aydin *et al*. 2007; Dongmei *et al*. 2008).

The aim of this study was to determine the potential nutritive value of a wild narrow-leaved clover (*Trifolium angustifolium)* hay harvested at 3 maturity stages using chemical composition and the *in vitro* gas production technique.

Materials and methods

Wild narrow-leaved clover plants were harvested at 3 maturity stages (2 weeks before flowering, at flowering and at seeding). The plants were hand-harvested from at least 3 replicate plots of

5 m x 2 m established in the experimental field. Samples were shade-dried and representative dry samples from each plot were taken to the laboratory and milled in a hammermill through a 1 mm sieve for subsequent analysis.

All chemical analyses were carried out in triplicate in the Department of Animal Science, Faculty of Agriculture, Kahramanmaras Sutcu Imam University, Turkey. Dry matter was determined by drying the samples at 105°C overnight and ash by igniting the samples in a muffle furnace at 525°C for 8 h. Nitrogen (N) concentration was measured by the Kjeldahl method (AOAC 1990) and converted to crude protein as N x 6.25. Neutral detergent fibre (NDF) was determined by the method of van Soest and Wine (1967) and ADF by the method of van Soest (1963). Condensed tannin concentration was determined by the butanol-HCl method as described by Makkar *et al*. (1995).

The milled samples were incubated *in vitro* with rumen fluid in calibrated glass syringes following the procedures of Menke *et al*. (1979) in the Department of Animal Science, Faculty of Agriculture, Uludag University, Bursa, Turkey. Rumen fluid was obtained from 3 fistulated sheep fed twice daily with a diet containing alfalfa hay (60%) and concentrate (40%). Samples (0.2 g) were weighed in triplicate into calibrated glass syringes of 100 mL. The syringes were prewarmed at 39°C before the injection of 30 mL rumen fluid-buffer mixture followed by incubation in a water bath at 39°C. Gas production was recorded at 3, 6, 12, 24, 48, 72 and 96 h after incubation. Total gas values were corrected for blank incubation. Cumulative gas production data were fitted to the exponential equation: $y = a + b\ (1\text{-}exp^{-ct})$ (Orskov and McDonald 1979), where: y is the gas production at time t; a is the gas production from the immediately soluble fraction (mL); b is the gas production from the insoluble fraction (mL); c is the gas production rate constant; a + b = the potential gas production (mL); and t = incubation time (h). Metabolisable energy (ME) concentration was calculated using the equation of Menke *et al*. (1979) as follows:

ME (MJ/kg DM) = 2.20 + 0.136 GP + 0.057 CP, where: GP = 24 h net gas production (mL/200 mg); and CP = crude protein.

Organic matter digestibility (%) of the forage samples was calculated using the equation of Menke *et al*. (1979) as follows:

OMD (%) = 14.88 + 0.889 GP + 0.45 CP +0.0651 XA where XA = ash concentration (%).

One-way analysis of variance (ANOVA) was carried out to determine the effects of maturity stage on the chemical composition and *in vitro* gas production kinetics of the forages using the General Linear Model (GLM) for Windows (1993). Significant differences between individual means at $P<0.05$ were identified using the Tukey's multiple range tests (Pearse and Hartley 1966). Standard errors of means were calculated from the residual mean square in the analysis of variance.

As a complement of ANOVA procedure, principal components analyses (PCA) were performed using chemical composition and *in vitro* gas production parameters as variables and maturity stage as classification criterion. The PCA analysis allows detection of the degree of association between variables by means of their relative position in a multivariate space, which is reduced to orthogonal directions of maximum variance in the original data (Afifi and Clark 1996). The biplot display proposed by Gabriel (1971) was

Table 1. Mean chemical composition of *Trifolium angustifolium* hay harvested at three maturity stages.

	Maturity stages				
	Pre-flowering	Flowering	Seeding	s.e.m	Sig.
		(%)			
DM[1]	92.7	93.0	92.4	0.10	NS
CP	16.9b[2]	13.7a	13.2a	0.31	***
NDF	43.7a	48.2b	51.5c	0.47	***
ADF	40.4a	43. 5b	45.4c	0.40	***
Ash	9.40a	11.96b	9.42a	0.08	***
CT	2.16a	2.39a	3.71b	0.18	***

[1] DM - dry matter; CP - crude protein; NDF - neutral detergent fibre; ADF - acid detergent fibre; CT - condensed tannin.

[2] Means within rows followed by a common letter do not differ (P>0.05).

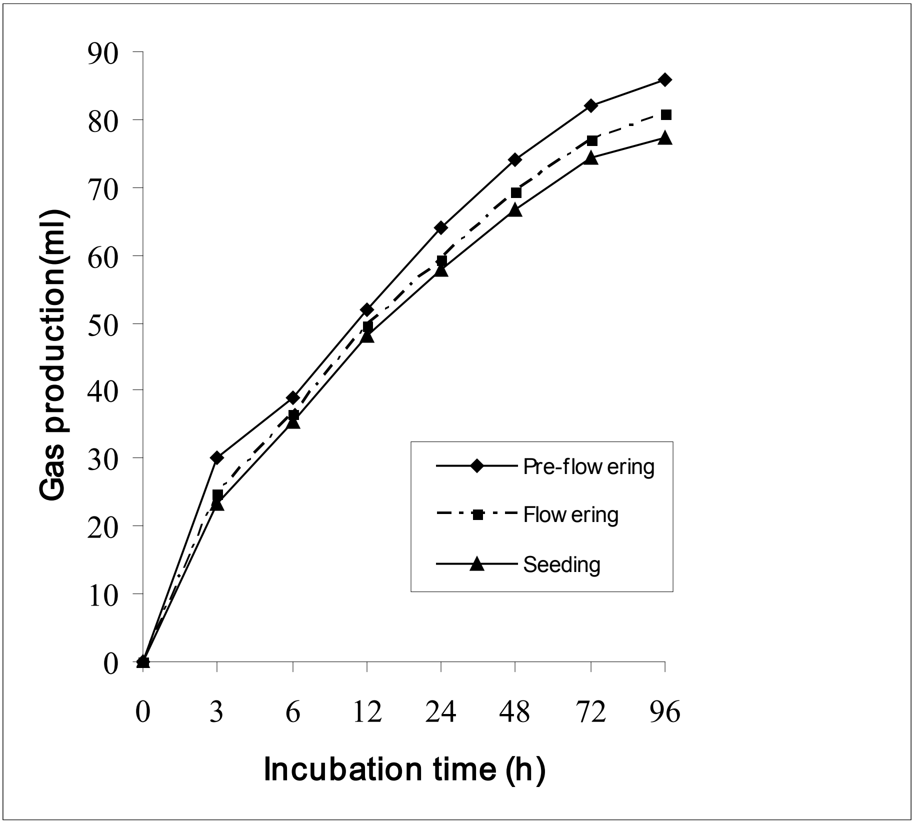

Figure 1. The effects of maturity stage on *in vitro* gas production from narrow-leaved clover.

used. Data were analysed using the SYSTAT 10 statistical software.

Results

The proximate chemical composition and condensed tannin concentrations in the forages were affected by maturity stage at harvest (Table 1). CP concentration declined from 16.9% pre-flowering to 13.2% at seeding ($P<0.05$). Both NDF and ADF concentrations increased ($P<0.05$) from pre-flowering to seeding (43.7 to 51.5% and 40.4 to 45.4%, respectively).

The ash concentration at flowering was significantly ($P<0.001$) higher than those at pre-flowering and seeding stages. Condensed tannin concentrations increased ($P<0.05$) from 2.16% pre-flowering to 3.71% at seeding.

Maturity stage had a significant effect on gas production from the hays (Figure 1), with a reduction in gas production with increasing maturity.

While stage of maturity had no significant ($P>0.05$) effect on the gas production rate (c) from the forages (Table 2), gas production from the immediately soluble fractions (a), and from the insoluble fraction (b) and potential gas production (a+b) decreased ($P<0.05$) with increasing maturity. Increasing maturity also resulted in a reduction in both ME concentration and OMD of the forages (Table 2).

The analysis of the first two principal components using the maturity stage classification criterion is given in Figure 2. These first two components explained 90.5% of the total variation. The variables CP, NDF, ADF, CT, a, b, a+b, ME and OMD showed the highest factor loading (0.946, -0.974, -0.944, -0.876, 0.892, 0.953, 0.993, 0.947 and 0.954, respectively), explaining 70.2% of the variation among maturity stages through the first component (PC I). The vari-

Table 2. The effects of maturity stage on *in vitro* gas production kinetics and some estimated parameters of *Trifolium angustifolium* hay when incubated with buffered rumen fluid.

	Maturity stage				
	Pre-flowering	Flowering	Seeding	s.e.m	Sig.
c[1]	0.085	0.084	0.087	0.002	NS
a	5.98b[2]	4.73a	3.96a	0.441	***
b	73.81c	70.49b	68.21a	0.373	***
a+b	79.80c	75.22b	72.17a	0.467	***
ME	11.87b	11.03ab	10.82a	0.066	***
OMD	80.0c	74.4b	72.8a	0.44	***

[1]c - gas production rate (%); a - gas production (mL) from quickly soluble fraction; b - gas production (mL) from the insoluble fraction; a+b - potential gas production (mL); OMD - organic matter digestibility (%); ME - metabolisable energy (MJ/kg DM).

[2]Means within rows followed by a common letter do not differ (P>0.05).

ables DM, ash and c showed the highest factor loading (-0.829, -0.845 and 0.772, respectively), explaining 20.33% of the variation through the second component (PC II). As can be seen from Figure 2, stage BF could be characterised by high OMD, ME, CP, b, a+b and stage S by high NDF, ADF and CT contents. On the other hand, stage F could be characterised by high ash concentration.

Discussion

This study has provided useful information on the likely decline in nutritive value of *Trifolium angustifolium* with increasing maturity. As has normally been found with plant species, crude protein concentration and digestibility declined with age, while NDF and ADF concentrations increased. Buxton (1996) reported that the decline in protein concentration with increasing maturity was a reflection of a decrease in protein in leaves, while stems, which had a lower protein concentration, represented a larger proportion of the available herbage in more mature forages. Minson (1990) reported that the average decreases in crude protein concentration with advancing maturity for several forages averaged 1 g/kg/day. In our case, the reduction from pre-flowering to seeding was 0.82 g/kg/d.

The CP concentrations measured in the current experiment were considerably lower than those reported by Tekeli *et al*. (2005) of 22.1 and 25.6% for forage harvested between pre-bloom and flowering stages. The plant we studied was a wild form of *Trifolium angustifolium,* whereas Tekeli *et al*. (2005) studied the cultivated form. The differences in CP concentrations between the studies could have been partly a response to genetic improvement in the cultivated form of *Trifolium angustifolium*.

The reduction in gas production with increasing maturity was also a reflection of declining quality and was in agreement with the findings of Kamalak *et al*. (2005a; 2005b), who reported a decrease in gas production as the forage growing period was prolonged.

With increasing maturity, the cell wall contents (NDF and ADF) increased, and these components were the more indigestible fractions of the plant. As a result, there was reduced gas production from the insoluble fraction (b) with increasing maturity. With the reduction in CP concentration and increase in the fibre fraction, OMD and ME concentration also decreased with increasing maturity. Figure 2 indicates that NDF and ADF concentrations were significantly and negatively correlated with OMD and ME, which is in agreement with the earlier findings of Kamalak *et al*. (2005a; 2005b).

The principal component analysis allowed better understanding of the complex correlations among the parameters related to chemical composition and *in vitro* gas production kinetics of *Trifolium angustifolium* hay. It also allowed discrimination among maturity stages in terms of chemical composition and *in vitro* gas production kinetics.

In ruminants, low levels of dietary condensed tannins (2–3%) can impart beneficial effects through reduced protein degradation in the rumen as a result of the formation of protein-tannin complexes (Barry 1987). However, optimal utilisation of CP could be restricted by high levels of condensed tannin (50 g/kg DM) owing to excessive formation of tannin-protein complexes, *e.g.* with endogenous protein, which pass through

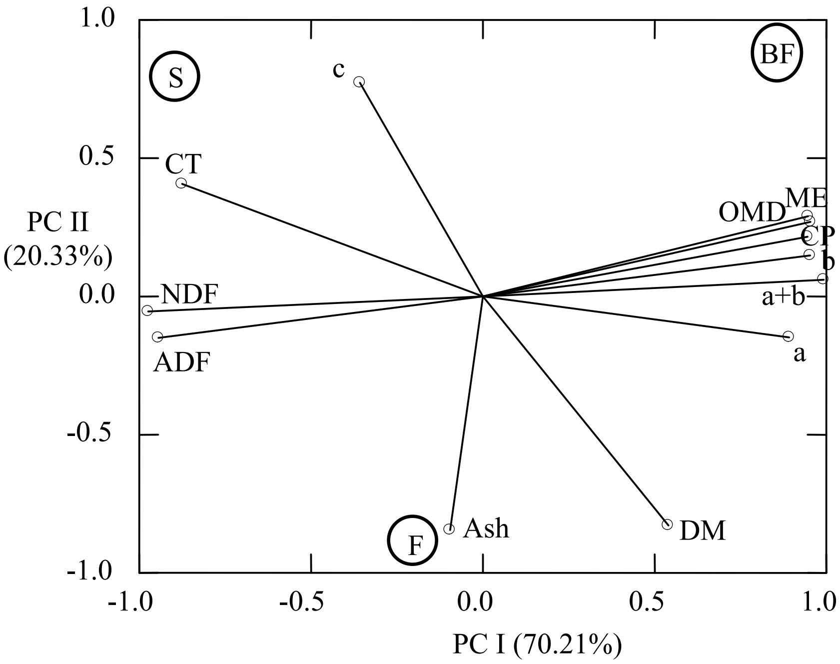

Figure 2. The analysis of the first two principal components using maturity stage classification criterion.

the animal largely undigested (Kumar and Singh 1984). Tannins can adversely affect the microbial and enzyme activities (Singleton 1981; Lohan *et al*. 1983; Barry and Duncan 1984; Makkar *et al*. 1989). However, in the current experiment, the condensed tannin levels of *Trifolium angustifolium* hay were lower than those considered detrimental to ruminant animals.

Conclusion

Despite the decline in nutritive value of the forage of *Trifolium angustifolium* with advancing maturity, even at the seeding stage, the forage had moderately high CP concentration and was quite digestible. It appears that wild *Trifolium angustifolium* could be harvested at these more advanced stages and still provide hay of an acceptable quality for ruminant animals during the winter period. The low levels of condensed tannins in the forage suggest it could be used as an alternative legume to reduce the risk of bloat in ruminants grazed on pastures. However, this would require verification with further testing.

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