Nutritive value of *Trifolium pratense* L. for ruminants estimated from *in situ* ruminal degradation of neutral detergent fibre and *in vivo* digestibility of organic matter and energy

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ABSTRACT: Seven clover (*Trifolium pratense* L.) samples were collected at three different stages of the same sward (first growth (I), $n = 3$; first regrowth (II), $n = 3$; second regrowth (III), $n = 1$) during the growing season from 10th of May to 17th of August. Samples were analyzed for chemical composition, gross energy (GE) content, *in vivo* organic matter digestibility (OMD) and gross energy digestibility (GED) in sheep, and *in situ* rumen degradability of neutral detergent fibre (NDF). The contents of ash, crude protein (CP), crude fibre (CF), NDF, acid detergent fibre (ADF), acid detergent lignin (ADL) and GE were significantly ($P < 0.05$) affected by the time of cutting. Average values of 119.2, 197.7, 214.1, 400.7, 296.2, 73.8 g/kg of dry matter (DM) and 18.2 MJ/kg of DM were obtained for ash, CP, CF, NDF, ADF, ADL and GE, respectively. In general, OMD and GED decreased as the cutting time progressed, with average values of 72.4% and 70.2%, respectively. Effective ruminal degradability (ED) of NDF generally decreased ($P < 0.05$) with the increasing date of cutting at each stage, with the values 66.1% (May 10), 63.6% (May 18), 59.2% (May 25), 64.8% (June 29), 57.4% (July 7), 56.9% (July 13) and 51.6% (August 17). *In situ* measurements were characterised by an average value of 77.1% for the fraction of NDF potentially degradable in the rumen (b), 0.0703 h^{-1} for the rate constant of disappearance of fraction b (c), and 77.7% for digestible NDF (DNDF).

Keywords: clover; forage; vegetation stage; NDF; indigestible NDF; potential degradability

Clover continues to be important forage for ruminants in the Czech Republic. Red clover is a common forage species, generally grown in mixed leys with large variation in the proportion of legumes and grasses (Rinne and Nykänen, 2000).

Information on ruminal degradability of feeds is crucial for effective diet formulation. Quantitative data on ruminal degradability of forages may be useful to characterise the nutritional value of feeds (Messman et al., 1996). A feed ration with an optimal concentration of structural fibre is an important regulator of feed intake, ruminal fermentation process, nutrient digestibility, health, animal production efficiency and profitability. To keep rumen fermentation balanced, high-producing dairy cows require a source of structural fibre to give integrity to the rumen contents, stimulate cud-chewing and produce fermentation end-products that can be used by the cows' tissues to produce milk fat and protein (Robinson and Putnam, 1999).

The above plant/animal interactions can be studied *in vivo*, *in situ*, and by other techniques.

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However, of all available analyses, forage quality is adequately predicted by neutral detergent fibre (NDF), since NDF is related to intake, rumination and cud-chewing stimulus, as well as to the energy value of forage. Taken together, these characteristics define the key aspects of forage quality (Robinson and Putnam, 1999).

A number of papers have concentrated on *in situ* analyses as the method for determination of degradability parameters of dry matter (DM), organic matter (OM), protein, fibre, minerals and other nutrients of feeds (e.g. Čerešňáková et al., 2007; Homolka et al., 2008; Jančík et al., 2008, 2009).

Feed quality, and therefore ruminal degradability, is influenced by several factors, with the most important stage of maturity of forage, forage species, environmental effects (location in combination with temperatures and precipitation), agronomic management, site of growth, and processing such as treatment and preservation (Pozdíšek and Vaculová, 2008; Tyrolová and Výborná, 2008; Jančík et al., 2009). Different patterns of maturation in forage species have a profound effect on animal performance in ruminants (Hetta et al., 2004). Furthermore, the protein value of forages is related to the stage of maturity (Písaříková et al., 2007) due to its influence on microbial synthesis and site of digestion (Gonzáles et al., 2001), with ruminal protein degradability decreasing as lucerne matures (Homolka et al., 2008).

The structure of forages is a critical precondition of their utilization by animals. Carbohydrates represent the largest nutrient component in dairy cow diets (Stokes, 1997). Neutral detergent soluble carbohydrates vary in ruminal fermentation pattern, end products and potential to yield microbial cells. Soluble fibre tends to produce more acetate, whereas the fermentation of sugars and starch will produce more propionate (Asadi Alamouti et al., 2009). There exist large differences between forages in the cell wall content and their individual fractions (cellulose, hemicellulose and lignin) which affect the rumen degradability of available nutrients (Čerešňáková et al., 2007).

This study, based on the current feed evaluation system, aimed to relate clover forage quality to digestive tract functions of ruminants. The nutritional value of forage (*Trifolium pratense* L.) was estimated by chemical analyses, *in vivo* organic matter digestibility and *in situ* NDF ruminal degradability methods. It was hypothesized that degradability of NDF and organic matter digestibility would decrease as maturing proceeded.

MATERIAL AND METHODS

Samples

Clover (*Trifolium pratense* L., Kvarta variety) forage was evaluated at three different stages of the same sward. The first (spring) growth (I) and first regrowth (II) included samplings on May 10, May 18 and May 25, and June 29, July 7 and July 13, respectively. The second regrowth (III) was represented by one date (August 17) of forage sampling. A description of sampling times and conditions is indicated in Table 1.

Table 1. Sampling of clover (*Trifolium pratense* L.) for evaluation

Sample	Stage	Average monthly temperature/rainfall	Botanical phenophases
1 (May 10)	I		young growth; standing height 25–30 cm
2 (May 18)	I	16.2°C/66 mm	young growth; standing height 30–35 cm; formation of flower buds
3 (May 25)	I		standing height 60 cm; end of flower budding up to beginning of blooming
4 (June 29)	II	16.5°C/98 mm	standing height 60 cm; beginning of blooming
5 (July 7)	II	17.2°C/103 mm	standing height 70 cm; beginning of blooming up to full blooming
6 (July 13)	II		standing height 70 cm; full blooming
7 (August 17)	III	17.7°C/57 mm	standing height 50 cm; after blooming

I = first (spring) growth; II = first regrowth; III = second regrowth

The chopped fresh material was dried at 50°C according to Harazim et al. (1999). Dried material was subsequently milled to pass through a one-millimetre sieve for chemical analyses and two-millimetre sieve for *in situ* determination. Fresh clover forages were fed to animals (wethers) for *in vivo* determination of digestibility in sheep.

Chemical analyses

Samples were analysed for contents of DM, ash, crude protein (CP), ether extract (EE), crude fibre (CF), NDF, acid detergent fibre (ADF) and acid detergent lignin (ADL). Ash-free concentrations of NDF, ADF and ADL were determined according to the methods described by Van Soest et al. (1991). The cell wall subcomponents (cellulose, hemicellulose and lignin) were calculated according to Van Soest et al. (1991) as follows: cellulose = ADF – ADL, hemicellulose = NDF – ADF and lignin = ADL. CP was analysed according to the Kjeldahl method (nitrogen × 6.25). EE was determined using Soxtec extraction with petroleum ether, and CF according to the AOAC (2005). Ash was determined after 4.5 h of combustion at 550°C (AOAC, 2005). Nitrogen-free extract (NFE) was calculated as DM – (CP + CF + EE + Ash). Total heating value (gross energy; GE) was measured using a calorimeter (IKA C 5000 control, IKA-Werke GmbH and Co. KG, Staufen, Germany).

In vivo analysis

At each sampling time chopped fresh clover samples were frozen until the *in vivo* trials (n = 7). The *in vivo* metabolic trials were performed on four wethers (Merino breed, live weight 83 ± 9 kg) stabled in balance crates according to Vencl (1985). The duration of 17 days for each trial was divided into two periods: 10 days for the adjustment and 7 days for the main experimental period. The feed ration was offered twice a day, at 6 a.m. and at 6 p.m. The animals had free access to drinking water.

During the main *in vivo* experimental period, feed intake and the amount of residual feedstuff and faeces were measured on a daily basis. The *in vivo* digestibility of nutrients in sheep was calculated as:

$$\text{Digestibility} = ((A - B)/A)$$

where:

A = average daily intake of nutrients

B = average quantity of undigested nutrients excreted

Digestible energy (DE) was calculated according to Sommer et al. (1994) from the following equation:

$$\text{DE (MJ/kg)} = \text{GE} \times \text{coefficient of } \textit{in vivo} \text{ digestibility of energy in sheep}$$

In situ analysis

The NDF degradability parameters (parameter b = fraction of NDF potentially degradable in the rumen; parameter c = rate constant of disappearance of fraction b, ED = effective ruminal degradability of NDF fraction assuming a fractional rate of passage to be 0.02 h^{-1}) were estimated by the *in situ* method. Nylon bags (pore size 42 microns, internal dimensions 50 × 120 mm with 30% open bag area, i.e. 15 mg of materials per 1 cm^2 of active free space; Uhelon 130 T, Silk and Progress Moravská Chrastová) were attached to a cylindrical carrier (Třináctý et al., 1996) and incubated in the rumen of three dry Holstein cows fitted with a rumen cannula for 2, 4, 8, 16, 24, 48, 72, 96 h (Harazim et al., 1999; Hvelplund and Weisbjerg, 2000) and 288 h (Rinne et al., 1999). Test samples were incubated in the rumen of each cannulated cow (two nylon bags per sample, incubation interval and cow). Individual animals were fed 4 kg of meadow hay, 10 kg of maize silage, 1 kg of barley meal and 0.1 kg of vitamin-mineral supplement daily. To produce an adequate amount of residues for Ankom fibre filter bag analysis, the sample sizes weighed into the nylon bags were: 0.50 g for 0, 2 and 4 h incubations; 0.75 g for 8 h incubation and 1.00 g for 16, 24, 48, 72, 96 and 288 h incubations, accepting that this would result in a variable initial sample-to-bag surface ratio. All nylon bags were placed into the rumen at 7 a.m., just before the morning feeding, and withdrawn according to the incubation schedule. Thereafter, nylon bags were rinsed, frozen, thawed and washed in running cold tap water for 20 min (bags were washed until no colour appeared in the water), and transferred with water to Ankom filter bags (Anonymous, 1998). Subsequently the residues in the Ankom filter bags were deep frozen (–18°C)

until NDF analysis. Before NDF boiling the Ankom fibre bags with residues were thawed and analysed for NDF content using an Ankom fibre analyser (Anonymous, 1998). The NDF boiling procedure should remove microbial matter. Sodium sulphite and heat stable α-amylase (Anonymous, 1998) were used to dissolve protein and starch, and ash-free NDF residues were subsequently determined after overnight drying at 100°C and combustion at 525°C (Koukolová et al., 2004).

The loss of small particles was estimated as 0 h incubation when the nylon bags were washed only with running cold tap water for 20 min. The obtained values for NDF degradability characteristics were corrected for the initial loss of small particles as described by Hvelplund and Weisbjerg (2000). *In situ* NDF degradability data were fitted to the exponential equation according to Ørskov and McDonald (1979):

$$\text{Deg}\ (t) = b \times (1 - \exp^{-ct})$$

$$\text{ED} = b \times (c/(c + k))$$

where:
Deg = disappearance rate at time t
ED = effective ruminal degradability of NDF
b = fraction of NDF potentially degradable in the rumen
c = rate constant of disappearance of fraction b
t = time of incubation
k = outflow rate of the rumen (k = 0.02/h)

In this study the estimated lag time (lt) was not included in the ED model due to the absence of any difference when included compared to exclusion. The prolonged incubation (288 hours) period was included to determine the *in situ* indigestible NDF (INDF), as described in methods used by Rinne et al. (1997). Indigestible NDF was calculated according to the equation of Lund (2002):

$$\text{INDF} = 100 - \text{DNDF}$$

where:
INDF = indigestible NDF (%)
DNDF = digestible NDF determined after 288 h incubation period (%)

Table 2. Chemical composition (g/kg of dry matter) and neutral detergent fibre cell wall composition of clover (*Trifolium pratense* L.) samples

Sample	1 (May 10)	2 (May 18)	3 (May 25)	4 (June 29)	5 (July 7)	6 (July 13)	7 (August 17)
Stage	I	I	I	II	II	II	III
Chemical composition (g/kg of dry matter)							
Original DM	154.7	190.1	180.3	137.3	159.4	157.0	241.0
Ash	145.3	103.6	91.2	135.2	98.8	139.0	121.6
EE	24.2	24.1	22.3	22.7	23.7	22.2	23.5
CP	218.8	211.1	179.9	213.9	197.6	181.6	180.7
CF	181.5	181.4	218.8	202.9	230.0	236.4	247.6
NDF	377.5	375.1	420.9	395.4	408.2	411.4	416.7
ADF	258.0	276.8	310.0	272.3	296.2	307.7	352.2
ADL	66.7	70.1	77.8	67.1	83.1	62.5	89.2
NFE	430.2	479.8	487.8	425.3	449.9	420.8	426.6
NDF cell wall composition (%)							
Cellulose	50.6	55.1	55.2	51.9	52.2	59.6	63.1
Hemicellulose	31.7	26.2	26.3	31.1	27.4	25.2	15.5
Lignin	17.7	18.7	18.5	17.0	20.4	15.2	21.4

ADF = acid detergent fibre; ADL = acid detergent lignin; CF = crude fibre; CP = crude protein; DM = dry matter; EE = ether extract; NDF = neutral detergent fibre; NFE = nitrogen free extract
The values are arithmetic means (n = 2) on a dry matter basis

Statistical analysis

Results were evaluated by the General Linear Model (GLM) procedure of SAS (SAS Institute, 2003). The ruminal degradability parameters *b* and *c* were estimated by the Non Linear Model (NLIN) procedures, and correlation coefficients between variables were computed using PROC CORR. Treatments were separated into main effect means for sampling (date of sampling which refers to growth number, i.e. May 10, May 18, May 25, June 29, July 7, July 13 and August 17) using the Scheffe's pairwise comparisons test. Statistical significance was declared at $P < 0.05$.

RESULTS AND DISCUSSION

The chemical composition of clover samples are presented in Table 2. The original DM varied between 137.3 (clover 4) and 241.0 (clover 7) g/kg of DM. Average values of 119.2 for ash, 23.2 for EE, 197.7 for CP, 214.1 for CF and 445.8 g/kg of DM for NFE were obtained. A decreasing CP content with increasing maturity is in accordance with trends reported by Rinne and Nykänen (2000). Fibre fractions, i.e. NDF, ADF and ADL, presented averages of 400.7, 296.2 and 73.8 g/kg of DM, respectively (Table 2). An increased amount of NDF, ADF and ADL within the observed stages is in accordance with Coblentz et al. (1998) and Elizalde et al. (1999). The detergent analysing system employed in the current study is used for determination of the insoluble cell wall matrix and estimation of its major subcomponents: cellulose, hemicellulose and lignin (Van Soest, 1994). Cellulose, hemicellulose and lignin were used to characterise the physical properties of the fibre the cell wall subcomponents (Table 2). NDF contained, on average, 55.4% cellulose, 26.2% hemicellulose and 18.4% of lignin. Some physical characteristics, including physical density, hydration capacity, cation exchange and fermentation rate, which are not manifested in chemical fractions or analyses, might have a considerable influence on the quality of feeds and forages (Van Soest, 1994).

In vivo organic matter digestibility (OMD) and gross energy digestibility (GED) in sheep are indicated in Table 3. The *in vivo* OMD and GED results presented mean values of 72.4 and 70.2%, respectively. Table 4 shows evidence that the energy content (MJ/kg of DM) of clover samples varied with plant maturation. In agreement with results obtained by Arieli et al. (1999), GE values ranged from 17.6 to 18.9 MJ/kg of DM and DE from 11.8 to 13.6 MJ/kg of DM.

The *in situ* degradability of NDF (%) of clover samples is presented in Figure 1. The *in situ* de-

Table 3. *In vivo* digestibility (%) of organic matter and gross energy of clover (*Trifolium pratense* L.) samples in sheep

Sample	1 (May 10)	2 (May 18)	3 (May 25)	4 (June 29)	5 (July 7)	6 (July 13)	7 (August 17)
Stage	I	I	I	II	II	II	III
OMD	68.7	76.2	74.5	74.9	71.1	72.5	68.9
GED	65.3	73.8	71.6	72.1	69.8	70.7	68.2

GED = gross energy digestibility; OMD = organic matter digestibility

Table 4. Energy content (MJ/kg of dry matter) of clover (*Trifolium pratense* L.) samples

Sample	1 (May 10)	2 (May 18)	3 (May 25)	4 (June 29)	5 (July 7)	6 (July 13)	7 (August 17)
Stage	I	I	I	II	II	II	III
GE	18.1	18.4	18.0	17.6	18.9	18.0	18.4
DE	11.8	13.6	12.9	12.7	13.2	12.8	12.6

DE = digestible energy; GE = gross energy

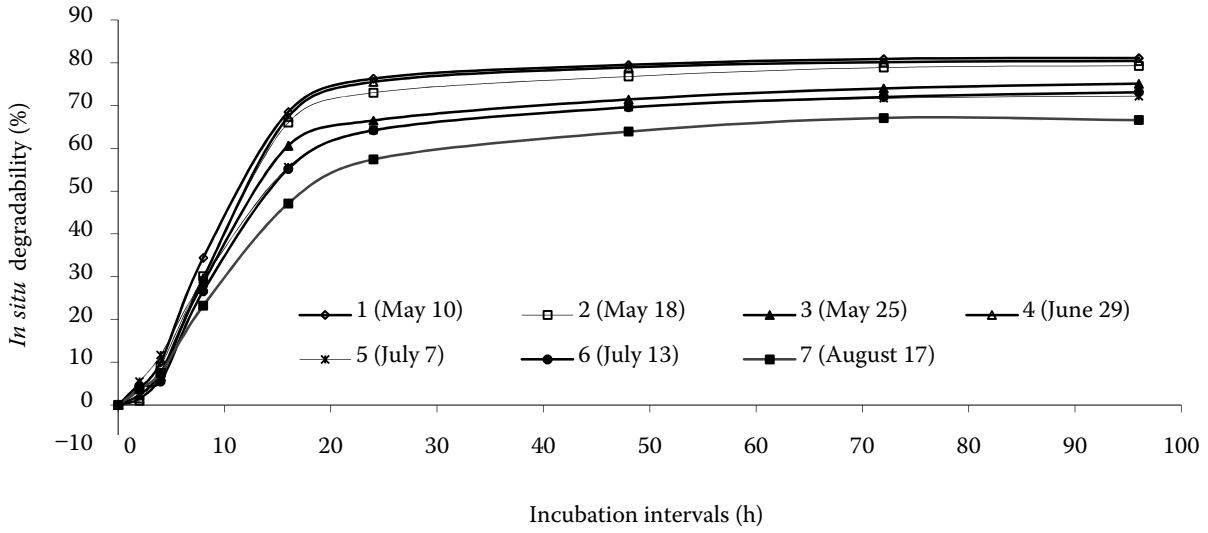

Figure 1. *In situ* degradability (%) of neutral detergent fibre (NDF) of clover (*Trifolium pratense* L.) samples

gradabilities of NDF after 24 h incubation were 76.3% (clover 1, May 10), 73.0% (clover 2, May 18), 66.5% (clover 3, May 25), 75.5% (clover 4, June 29), 64.2% (clover 5, July 7), 64.2% (clover 6, July 13) and 57.4% (clover 7, August 17). The indigestible NDF (INDF) content of clover varied between 17.0 and 30.4%. The effect of forage harvest time on NDF *in situ* degradability was noted in some earlier studies (Dehority, 1993; Rinne et al., 2002). Rinne et al. (2002) observed an increasing content of INDF with increasing maturity of forage, being 4.8, 5.7, 7.8 and 12.4% for forages harvested at four different times from June 13 to July 4, respectively.

Degradation kinetics and effective degradability (ED) calculated at a fractional passage rate of 0.02^{-1} are given in Table 5. The values of ED of NDF were on average 66.1% (May 10), 63.6% (May 18), 59.2% (May 25), 64.8% (June 29), 57.4% (July 7), 56.9% (July 13) and 51.6% (August 17). The *b* value, with an average value of 77.1%, was in general lower than digestible NDF (DNDF) measured after 288 h incubation (average value of 77.7%). The *c*-values ranged from 0.0621 to 0.0769 h^{-1}. The above results are in agreement with Koukolová et al. (2004), whereas the effects of maturity on degradability characteristics were comparable with those found in forage by Elizalde et al. (1999) and Yu et al. (2004). Differences in phenophases are related to cell wall structures, complexity and components such as phenolic acid and lignin concentrations (Yu et al., 2003, 2004), which are of vital importance for the cell wall carbohydrate degradability (Jung and Allen, 1995). Significant differences ($P <$

Table 5. The neutral detergent fibre (NDF) degradability characteristics of clover (*Trifolium pratense* L.) samples

Sample	1 (May 10)	2 (May 18)	3 (May 25)	4 (June 29)	5 (July 7)	6 (July 13)	7 (August 17)
Stage	I	I	I	II	II	II	III
b (%)	83.3^{a}	81.3^{a}	76.2^{b}	83.1^{a}	73.2^{c}	74.3^{b,c}	68.4^{d}
c (h^{-1})	0.0769	0.0725	0.0704	0.0710	0.0731	0.0666	0.0621
ED (%)	66.1^{a}	63.6^{b}	59.2^{c}	64.8^{a,b}	57.4^{c,d}	56.9^{d}	51.6^{e}
DNDF (%)	82.7^{a}	81.1^{a}	77.8^{b}	83.0^{a}	74.9^{c}	75.1^{c}	69.6^{d}

b = fraction of NDF potentially degradable in the rumen; *c* = rate constant of disappearance of fraction *b*; ED = effective ruminal degradability; DNDF = digestible NDF

^{a,b,c,d,e}means within rows with different superscripts differ ($P < 0.05$)

Table 6. Correlation coefficients between chemical composition (g/kg of dry matter), gross energy (MJ/kg of dry matter), NDF degradation characteristics (units of *b*, ED and INDF are in %, and *c* in h^{-1}) and digestibility (%) of organic matter

	Original DM	Ash	EE	CP	CF	NFE	NDF	ADF	ADL	GE	*b*	*c*	ED	INDF
Ash	–0.317													
EE	0.219	–0.001												
CP	–0.503	0.336	0.607											
CF	0.404	–0.134	–0.492	–0.861*										
NFE	0.166	–0.858*	0.063	–0.079	–0.300*									
NDF	0.300	–0.318	–0.688	–0.908*	0.904*	–0.031								
ADF	0.771*	–0.273	–0.324	–0.885*	0.880*	–0.069	0.808*							
ADL	–0.458	–0.572	0.201	–0.458	0.555	0.194	0.525	0.702						
GE	0.387	–0.535	0.546	–0.155	0.250	0.257	0.066	0.272	0.612					
b	–0.587	0.389	0.293	0.889*	–0.933*	0.027	–0.852*	–0.919*	–0.718	–0.506				
c	–0.474	–0.323	0.495	0.657	–0.758*	0.551	–0.659	–0.782*	–0.287	0.267	0.330*			
ED	–0.607	0.228	0.373	0.902*	–0.968*	0.174	–0.873*	–0.961*	–0.662	0.341	0.953*	0.599*		
INDF	0.683*	–0.205	–0.056	–0.757*	0.845*	–0.175	0.623	0.878*	0.640	0.595	–0.953*	–0.479*	–0.961*	
OMD	–0.268	–0.405	–0.356	0.107	–0.332	0.574	–0.137	–0.280	–0.361	–0.298	0.329	0.354	0.365	–0.442

ADF = acid detergent fibre; ADL = acid detergent lignin; *b* = fraction of NDF potentially degradable in the rumen; *c* = rate constant of disappearance of fraction *b*; CF = crude fibre; CP = crude protein; DM = dry matter; ED = effective ruminal degradability; EE = ether extract; GE = gross energy; INDF = indigestible NDF; NDF = neutral detergent fibre; NFE = nitrogen free extract; OMD = *in vivo* digestibility of organic matter

*statistical significance $P < 0.05$

0.05) among clover harvest times in the ED of NDF, *b* and DNDF were observed.

Correlation coefficients between chemical composition, GE, NDF degradability parameters (*b*, *c*, ED and INDF) and *in vivo* OM digestibility (OMD) are shown in Table 6. Strong correlations ($P < 0.05$) were observed between the ED NDF and CP ($r = 0.902$), CF (–0.968), NDF ($r = -0.873$), ADF ($r = -0.961$), *b* ($r = 0.953$) and *c* ($r = 0.599$). INDF was significantly ($P < 0.05$) related to the content of original DM ($r = 0.683$), CP ($r = -0.757$), CF ($r = 0.845$) and ADF ($r = 0.878$). The NDF degradability parameters *b*, *c* and ED showed significant ($P < 0.05$) correlation coefficients of –0.953, –0.479 and –0.961, respectively, with INDF.

The effect of declining feed digestibility with increasing maturity, as demonstrated by Rinne et al. (2002) and Jančík et al. (2008), was clearly illustrated in the present study. Micek et al. (2001) found a decrease in the DM degradability parameters *b* and *c* with increasing maturity of oats, whereas Jančík et al. (2008) confirmed a decrease in *b* and ED of NDF and an increase in INDF content after the start of phenophases of some grass species. Furthermore, Jančík et al. (2008) concluded that INDF content could be effectively predicted from ADL contents. INDF content markedly increased during forage maturation, which has practical implications for the time of harvest (Jančík et al., 2008).

CONCLUSIONS

The present study documents the variation in individual nutrient contents during a vegetation period. *In situ* NDF degradability parameters were related to individual nutrients, and *in vivo* digestibility of OM and GE in sheep to the time of forage harvest.

The fraction NDF potentially degradable in the rumen (*b*), rate constant of disappearance of fraction *b* (*c*) and effective ruminal degradability of NDF (ED) were related ($P < 0.05$) to indigestible NDF (INDF). Effective ruminal degradability of NDF (ED) was linearly reduced ($P < 0.05$) as the forage maturation increased. Considering the range of hemicelluloses and cellulose content in the samples with regard to lignin, the organic matter digestibility decreases with an increase in lignin content. This affects the diet composition and subsequently the level of feed intake, digestive processes and finally animal production together with the farm prosperity. The results take into account the utilization status in the digestive tract of ruminants, which is important for development of digestibility methods.

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