View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by AJOL - African Journals Online

South African Journal of Animal Science 2017, 47 (No. 1)

Short communication

Effects of drying procedures on chemical composition and nutritive value of alfalfa forage

F. Jančík[#], P. Kubelková, V. Kubát, M. Koukolová & P. Homolka
Institute of Animal Science, Přátelství 815, Prague-Uhříněves, 104 00, Czech Republic

(Received 8 October 2015; Accepted 10 November 2016; First published online 31 December 2016)

Copyright resides with the authors in terms of the Creative Commons Attribution 4.0 South African License.
See: http://creativecommons.org/licenses/by/4.0/za
Condition of use: The user may copy, distribute, transmit and adapt the work, but must recognize the authors and the South African Journal of Animal Science.

Abstract

The effects of various drying procedures of alfalfa forage were evaluated on chemical composition, *in vitro* neutral detergent fibre (NDF) and dry matter (DM) digestibility, *in situ* DM, organic matter and crude protein (CP) degradability. The alfalfa had been harvested in the spring growth (early bud and flowering) and first regrowth (late bud and late flowering) periods. The samples were dried at 30 °C (T30), 40 °C (T40), 50 °C (T50), 60 °C (T60) and 100 °C (T100) in a forced-air oven or frozen for one month and then freeze-dried (TFD) or oven-dried at 50 °C (TFREE). Another drying procedure included pre-treatment by heating in a microwave oven (TMO) or in a forced-air oven at 100 °C for 1 hour (T100+50) and then oven-dried at 50 °C. The freeze-drying method was chosen as a reference method. Freeze-dried samples had the lowest NDF, acid detergent fibre (ADF), acid detergent lignin (ADL), neutral detergent insoluble protein (NDIP) and acid detergent insoluble protein contents ($P < 0.05$). Additionally, freeze-dried products had the highest CP, *in vitro* true digestibility of DM and CP degradability values ($P < 0.05$). There was no added benefit of the TMO in the chemical composition, *in vitro* digestibility or in situ degradation compared with T50. This study showed that T50 can yield chemical composition, *in vitro* and *in situ* results that are similar to those obtained with the freeze-drying method and that this procedure is useful for forage analyses and evaluation.

Keywords: freeze-drying, insoluble nitrogen, *in vitro* digestibility, oven-drying, ruminants
[#] Corresponding author: jancik.filip@vuzv.cz

An accurate estimate of ingredient nutritional value is necessary for precise formulations of feed rations in all groups of farm animals, and useful in evaluating forages. The drying method can greatly affect the results of proximate analyses (Parissi *et al.*, 2005). Drying methods that involve heat may alter the chemical composition of feeds, including the NDF and lignin content, and may ultimately lead to the formation of indigestible Maillard products (Purcell *et al.*, 2011). Different drying methods could affect the content of chemical components, particularly the adhesion of nitrogen to NDF and ADF. They may also lead to an inaccurate determination of digestibility. Purcell *et al.* (2011) concurred and reported that freeze-drying (FD) should be considered an ideal system for drying samples. Compared with FD samples, Pelletier *et al.* (2010) detected lower nitrogen (N) and higher neutral detergent insoluble nitrogen (NDIN) concentrations when the forages were oven-dried. Parissi *et al.* (2005) and Pelletier *et al.* (2010) found a decrease in the *in vitro* digestibility values for forages that were dried in an oven compared with FD. However, Pelletier *et al.* (2010) described FD as a method that requires access to expensive equipment, and is difficult to apply to large samples under field conditions. Pelletier *et al.* (2010) therefore proposed microwave pre-treatment, a fast and easy procedure that can be applied to a wide range of forage species, as an alternative to FD when it is not feasible. To the knowledge of the authors, no studies have compared a high number of conventional oven drying (OD) and innovative drying procedures (pre-treatments by freezing, microwave heating or short drying at high temperature) with FD on samples taken from the same plant material. The objective of this paper was to study the effect of various drying procedures on the chemical composition, NDIN, acid detergent insoluble nitrogen (ADIN), and *in vitro* digestibility and *in situ* degradability of alfalfa.

Alfalfa (*Medicago sativa* L.; cv. Soča) was sown in 2011 on brown soil at Praha-Uhříněves, Czech Republic (50°2'N, 14°36'E). Tested samples were harvested on 16 May (early bud) and 8 June (flowering)

URL: http://www.sasas.co.za
ISSN 0375-1589 (print), ISSN 2221-4062 (online)
Publisher: South African Society for Animal Science
http://dx.doi.org/10.4314/sajas.v47i1.14

for spring growth and 20 June (late bud) and 7 July (late flowering) for the first regrowth in 2013. The samples were harvested in the morning (10:00) from three 10 m^2 areas that were divided into three replication sets of nine sub-samples (n = 108; 4 harvests × 9 drying treatments × 3 replicates). Each sub-sample was treated with one of the nine treatments, as described in Table 1. The same procedure was used for all four harvesting dates. Drying for the treatments was at 30 °C (T30), 40 °C (T40), 50 °C (T50), 60 °C (T60) and 100 °C (T100), and was done by placing the 1000 g forage samples in stainless steel pans (40 cm × 55 cm), which were then placed directly into a forced-air oven. Two sub-samples were pre-treated by freezing after being placed into a plastic bag (1000 g per sample) for one month at -20 °C. The frozen samples were subsequently removed from the plastic bags, put in stainless steel pans, and placed directly in the oven (TFREE) or freeze dryer (TFD) (freeze dryer Alpha 1-4 LSC, Martin Christ Gefriertrocknungsanlagen GmbH, Germany) without a thawing period. One sub-sample of each sample set was pre-treated by heating in a microwave oven (Fagor Mo25DGB); 250 g of sample was heated for 70 s at high intensity to reach approximately 70 °C and then dried at 50 °C for 48 h (TMO). One sub-sample of each sample set was pre-treated by heating in a forced-air oven at 100 °C for 1 h and then dried at 50 °C for 48 h (T100+50). Dried samples were ground (Retsch SM 100; Retsch GmbH, Haan, Germany) using a 2-mm sieve for *in situ* analysis and through a 1-mm sieve for chemical and *in vitro* analysis.

Table 1 Description of drying treatments applied to alfalfa samples

Treatment abbreviation	Drying pre-treatment	Drying treatment	n
TFD	Frozen in plastic bag at -20 °C for 1 month	Freeze-dried	12
T100	None	Dried at 100 °C for 24 h	12
T60	None	Dried at 60 °C for 48 h	12
T50	None	Dried at 50 °C for 48 h	12
T40	None	Dried at 40 °C for 72 h	12
T30	None	Dried at 30 °C for 96 h	12
T100+50	Dried at 100 °C for 1 h	Dried at 50 °C for 48 h	12
TMO	Heated in a microwave oven for 70 s at maximum intensity to reach approximately 70 °C	Dried at 50 °C for 48 h	12
TFREE	Frozen in plastic bag at -20 °C for 1 month	Dried at 50 °C for 48 h	12

DM was determined by oven drying for 6 h at 105 °C. The ash was determined after 6 h combustion at 550 °C. N was determined with the Kjeldahl method (Kjeltec Auto 1030 Analyser, Höganäs, Sweden), according to the AOAC official method 976.05 (AOAC, 2005). CP was calculated as N × 6.25. ADF and ADL contents were determined according to the AOAC official method 973.18 (AOAC, 2005). The NDF content of samples and digested residues was analysed in the presence of sodium sulphite following an α-amylase treatment (Van Soest *et al.*, 1991). It was presented ash-free. Fibre fractions were determined with ANKOM^{220} (ANKOM Technology, Macedon, NY, USA). NDIN and ADIN were determined according to the method described by Licitra *et al.* (1996). NDIP and acid detergent insoluble protein (ADIP) were calculated as NDIN or ADIN × 6.25 (presented in g/kg CP).

The protocol of the present experiment was approved by the Animal Care and Use Committee, Institute of Animal Science, Prague, Czech Republic (Act No. 359/2012 Coll.). The *in situ* method was used to determine the ruminal degradability of CP, organic matter (OM) and DM. All sub-samples were incubated in the rumen of two Holstein dairy cows for 24 hours. Approximately 5 g ground material was weighed into each bag (100 × 200 mm; pore size 50 µm), and 6 bags (three per cow) were prepared for each sample. Incubations were done in three runs. The cows were fed a diet in total mixed rations form in the amount of 45.95 kg per cow, comprising alfalfa silage (17 kg), maize silage (15 kg), maize LKS (3.5 kg), brewer's draft (7.5 kg), concentrate mixture (3 kg), mineral supplement (0.15 kg), wheat straw (0.30 kg) and alfalfa hay (0.50 kg). The diet was presented at 07:30 and 18:30 h (in proportions of 50 %). After removal from the rumen, the bags were plunged immediately into cold water and washed by hand for 30 minutes. After washing, the residues were quantitatively transferred to the plastic plates and dried for 48 hours at 50 °C. The residues were then weighed and analysed for CP, OM and DM. The *in vitro* true digestibility of DM (IVTD) and NDF (dNDF) was measured with an ANKOM Daisy II incubator (ANKOM Technology, Macedon,

NY, USA), as described by Damiran *et al.* (2008). *In vitro* incubations were performed in triplicate on each alfalfa sample in three runs. The IVTD and dNDF were calculated according to Pelletier *et al.* (2010).

The data were analysed using the MIXED procedure of SAS (SAS Institute Inc., 2002), with drying methods (n = 9), stage of development (n = 4) and their interaction as fixed effects. Replicates were considered a random effect (n = 3). The data obtained using the in situ method were analysed as described above. The model was supplemented by the cow (n = 2) as a repeated effect. Statistical significance was determined at P <0.05. Least square means were reported. Comparisons of least squares means were done with the Tukey-Kramer adjustment.

The TFD was chosen as a reference method because it yields dried samples that are more closely related to living tissues (Pagán *et al.*, 2009). The DM measured by TFD was comparable with that observed with other drying methods, except T40 and T60 (Table 2). The NDF content was higher when using the OD methods compared with the TDF (P <0.05). The highest value was detected for T100 (P <0.05). The TFD also yielded the lowest ADF and ADL contents, which were comparable with the TMO and T100+50 (Table 2).

CP content was lower (*P* <0.05) when T60, T100 and T100+50 were used than with TDF (Table 2). Compared with other drying methods, markedly higher NDIP content (Table 2) was detected for T100 and T100+50 (*P* <0.05). On the contrary, the TFD yielded the lowest numerical contents of NDIP. Compared with other methods, T100 yielded the highest ADIP content. The T100+50 yielded a higher ADIP content than TFD (*P* <0.05). The lower fibre and lignin concentrations observed in the FD forages compared with the OD samples had also been observed in previous studies. Cone *et al.* (1996) found that the NDF content in protein-rich samples differed after using the drying methods compared with TFD, because of proteins that were bound to the NDF matrix. Parissi *et al.* (2005) showed an increase in the NDF and ADL content of forages as a result of OD owing to the formation of insoluble condensed tannin-protein polymers. Additionally, the ADF could be increased by the formation of Maillard reaction products, which occurs when samples are dried at high temperatures (Pelletier *et al.*, 2010). Pagán *et al.* (2009) demonstrated that the complexes formed by the OD technique were not dissolved within detergent extractions. This information was confirmed by the results of the NDIP and ADIP, in which rapid increases in NDIP and ADIP contents were detected when the samples were treated to above 40 °C and 60 °C, respectively. Nishino *et al.* (1994) also described an increase in the ADIN content in alfalfa when it was heated to 60 °C. This happened because of non-enzymatic protein-carbohydrate reactions, which are regarded as not available for animals. Compared with FD, Alomar *et al.* (1999) detected a 0.5 % reduction in the CP content as a result of the OD (65 °C for 48 h) of silages. In the current study, the differences of CP between TFD and T60 and T100 were 10 and 16 g/kg CP, respectively. This was probably caused by losses of compounds such as ammonia and other non-protein nitrogenous volatile substances (Alomar *et al*., 1999), owing to high temperatures of T60 and T100.

The goal of utilising the short-time heating pre-treatments (T100+50 and TMO) was to increase quickly the temperature of the samples and rapidly remove moisture, while rapidly inhibiting enzyme activity and limiting the loss of carbohydrates (Pelletier *et al*., 2010). The T100+50 and TMO were both comparable with the TFD regarding ash, ADF and ADL contents. The TMO did not differ with TFD in CP and ADIP contents, but both methods differed from TFD in NDF and NDIP contents. Pelletier *et al.* (2010) also compared the short time high temperature and microwave pre-treatments with FD for alfalfa and found a difference only in the NDIN content; N, ADF, NDF and ADIN contents did not differ. The same authors showed a small effect of microwave pre-treatment on the ADIN concentration compared with the NDIN concentration, which suggests that this procedure affected the true protein (soluble and insoluble protein in neutral detergent) more than the indigestible protein (ADIP) (Licitra *et al.*, 1996).

The *in vitro* true digestibility of DM was higher (P <0.05) in the TFD than in T30, T40, T50, T60 and T100 (Table 3). Compared with TFD, only T40 yielded a lower in vitro NDF digestibility value (P <0.05). The DM degradability differed among TFD and T100, T40 and TMO by 26, 28 and 38 g/kg DM (*P* <0.05), respectively (Table 3). The values describing CP degradability (CPD) showed the impact of the drying temperature on CP utilisation. The lowest values of CPD were detected for T100, followed by T100+50, TMO and T60 (Table 3). TFD yielded the highest numerical value for CPD. These values corresponded to the values observed for the CP and NDIP content when the lowest CPD values were found for samples with the lowest CP content and the highest NDIP content. This study confirmed the results of Pelletier *et al.* (2010), who showed that IVTD tended to be higher in the FD and TMO samples than in the samples subjected to the other drying procedures. This finding suggests that leguminous species should be dried at low temperatures to avoid protein denaturation and to prevent fermentation. In the present study, no differences (*P* >0.05) were observed in the results of in vitro and in situ methods for TFREE, TFD, and T50. Huntington & Givens (1997) reported a reduction of DM degradability of pre-frozen grass silages. The authors also reported that freezing storage could form ice crystals in plant cell membranes. This could initiate or enhance subsequent

Table 2 Effect of drying method on the chemical composition (g/kg) of alfalfa

Item	M	Drying method*												P value		
		TFD	T100	T60	T50	T40	T30	T100+50	TMO	TFREE	Mean	n	SEM	D	M	D×M
DM	EB	175^{J}	169^{JK}	166^{KL}	161^{KL}	159^{L}	163^{KL}	166^{KL}	162^{KL}	167^{JKL}	165^{z}	27	1.5	**	**	**
	LB	203^{GH}	205^{G}	203^{GH}	200^{GH}	191^{I}	196^{HI}	202^{GH}	201^{GH}	207^{G}	201^{y}	27				
	F	232^{CDE}	232^{CDE}	218^{F}	240^{ABC}	225^{EF}	236^{BCD}	231^{CDE}	235^{BCD}	229DE	231^{x}	27				
	LF	241^{AB}	242^{AB}	238^{ABC}	239^{ABC}	237^{BC}	235^{BCD}	247^{A}	241^{AB}	242^{AB}	240^{w}	27				
	Mean	213^{a}	212^{ab}	206^{bc}	210^{ab}	203^{c}	208^{abc}	211^{ab}	210^{ab}	211^{ab}		108				
Ash	EB	103^{F}	106^{E}	112^{BC}	109^{D}	118^{A}	114^{B}	111^{C}	110^{CD}	107^{E}	110^{w}	27	0.5	**	**	**
	LB	98^{G}	90^{KL}	90^{KL}	90^{KL}	98^{G}	95^{H}	91^{JK}	96^{GH}	92^{IJ}	93^{x}	27				
	F	97^{G}	89^{L}	97^{G}	94^{HI}	97^{G}	92^{IJ}	95^{H}	91^{JK}	89^{L}	93^{x}	27				
	LF	82^{M}	74^{O}	78^{N}	79^{N}	80^{MN}	74^{O}	78^{N}	79^{N}	80^{MN}	78^{y}	27				
	Mean	95.1^{ab}	89.6^{c}	94.2^{b}	93.2^{bc}	98.5^{a}	93.6^{b}	93.8^{b}	94.2^{b}	91.8^{bc}		108				
NDF	EB	335^{OP}	374^{M}	337^{O}	336^{O}	332^{OP}	352^{N}	321^{P}	333^{OP}	321^{P}	338^{z}	27	2.5	**	**	**
	LB	359^{N}	449^{FG}	428^{HIJ}	428^{HIJ}	421^{IJK}	423^{IJK}	407^{L}	418^{JKL}	412^{KL}	416^{y}	27				
	F	421I^{JK}	454^{EFG}	459^{EF}	432^{HI}	449^{FG}	458^{EF}	441^{GH}	425^{IJK}	455^{EFG}	444^{x}	27				
	LF	460^{EF}	512^{A}	486^{BC}	480^{CD}	480^{CD}	496^{B}	467DE	469DE	461^{EF}	479^{w}	27				
	Mean	394^{d}	447^{a}	427^{b}	419^{bc}	420^{bc}	432^{b}	409^{c}	411^{c}	412^{c}		108				
ADF	EB	274^{L}	279^{L}	272^{L}	265^{LM}	270^{L}	281^{L}	252^{M}	271^{L}	270^{L}	270^{z}	27	2.9	**	**	**
	LB	299^{K}	361^{GH}	358^{GHI}	349^{HIJ}	347^{HIJ}	353^{HIJ}	341^{J}	339^{J}	347^{HIJ}	344^{y}	27				
	F	344^{IJ}	371^{FG}	381^{EF}	337^{J}	383^{EF}	381^{EF}	363^{GH}	351^{HIJ}	386^{DEF}	366^{x}	27				
	LF	380^{F}	419^{A}	410^{AB}	393^{CDE}	409^{ABC}	402^{BCD}	386^{DEF}	383^{EF}	386^{DEF}	397^{w}	27				
	Mean	324^{c}	357^{a}	355^{a}	336^{bc}	352^{a}	354^{a}	336^{bc}	336^{bc}	347^{ab}		108				
ADL	EB	37.2^{P}	48.1^{N}	41.8^{OP}	43.2^{NO}	42.4^{OP}	46.4^{NO}	37.2^{P}	37.6^{P}	37.5^{P}	41.3^{z}	27	0.9	**	**	**
	LB	58.2^{M}	71.4^{DEF}	66.9^{F-J}	63.1^{I-M}	67.0^{F-J}	66.3^{F-J}	61.3^{KLM}	63.4^{I-M}	62.5^{J-M}	64.5^{y}	27				
	F	61.0^{LM}	64.5^{H-L}	66.4^{F-K}	65.5^{G-L}	70.7^{EFG}	68.4^{F-I}	64.9^{H-L}	62.7^{J-M}	69.5^{FGH}	66.0^{x}	27				
	LF	75.4^{DE}	88.2^{A}	80.8^{BC}	81.1^{BC}	82.7^{B}	83.5^{AB}	79.3^{BCD}	77.2^{CD}	76.5^{CD}	80.5^{w}	27				
	Mean	58.0^{f}	68.1^{a}	64.0^{bcd}	63.2^{cde}	65.7^{abc}	66.2^{abc}	60.7^{ef}	60.2^{ef}	61.5^{de}		108				
CP	EB	220^{BC}	204^{FG}	212DE	224^{AB}	226^{A}	224^{AB}	208^{EF}	212DE	219^{BC}	217^{w}	27	1.2	**	**	**
	LB	217^{CD}	191^{I}	200^{GH}	198^{H}	201^{GH}	211^{E}	198^{H}	200^{GH}	201^{GH}	202^{x}	27				
	F	169^{KL}	161^{MNO}	163^{MN}	170^{K}	170^{K}	165^{LM}	156^{O}	173^{JK}	163^{MN}	166^{z}	27				
	LF	171^{K}	157^{O}	161^{MNO}	169^{K}	172^{JK}	160^{N}	177^{J}	168^{KL}	172^{JK}	167^{y}	27				
	Mean	194^{a}	178^{c}	184^{bc}	190^{ab}	192^{a}	190^{ab}	185^{b}	188^{ab}	189^{ab}		108				
NDIP	EB	63.2^{R}	464^{A}	104^{NOP}	106^{NOP}	89.5^{PQ}	94.7^{OPQ}	412^{CD}	141^{JK}	118^{LMN}	177^{z}	27	3.2	**	**	**
	LB	81.7^{Q}	400^{D}	127^{KL}	154^{IJ}	109^{MNO}	103^{NOP}	439^{B}	301^{E}	170^{HI}	209^{x}	27				
	F	96.7^{OPQ}	314^{E}	141^{JK}	173^{H}	124^{KLM}	119^{LMN}	428^{BC}	265^{F}	166^{HI}	203^{y}	27				
	LF	130^{KL}	395^{D}	161^{HI}	170^{HI}	131^{KL}	154^{IJ}	427^{BC}	302^{E}	197^{G}	230^{w}	27				
	Mean	92.9^{e}	393^{a}	133^{cd}	151^{cd}	113de	118de	426^{a}	252^{b}	163^{c}		108				
ADIP	EB	55.4^{MNO}	195^{A}	60.7^{J-O}	49.3^{NO}	61.8^{I-N}	54.6^{MNO}	64.8^{H-N}	59.6^{K-O}	51.8^{NO}	72.5^{y}	27	3.2	**	**	**
	LB	43.1^{O}	99.0^{BCD}	59.7^{K-O}	59.2^{L-O}	71.2^{G-M}	62.5^{H-N}	92.7^{C-F}	66.4^{G-N}	76.2^{F-L}	70.0^{y}	27				
	F	58.1^{MNO}	101^{BCD}	77.5^{F-J}	70.8^{G-M}	72.3^{G-M}	75.3^{F-L}	92.1^{C-F}	70.3^{G-M}	79.8^{E-H}	77.4^{x}	27				
	LF	77.2^{F-K}	83.6^{D-G}	79.0^{E-I}	107^{BC}	84.0^{D-G}	91.7^{C-F}	95.2^{B-E}	111^{B}	106^{BC}	92.8^{w}	27				
	Mean	58.5^{c}	119.5^{a}	69.2^{bc}	71.7^{bc}	72.3^{bc}	71.0^{bc}	86.2^{b}	76.8^{bc}	78.5^{bc}		108				

*TFD: Freeze-dried; T100: 100 °C for 24 h; T60: 60 °C for 48 h; T50: 50 °C for 48 h; T40: 40 °C for 72 h; T30: 30 °C for 96 h; T100+50: 50 °C for 48 h; TMO: 50 °C for 48 h; TFREE: 50 °C for 48 h; **: *P* <0.01; SEM: standard error of mean. DM: dry matter; NDF: neutral detergent fibre; ADF: acid detergent fibre; ADL: acid detergent lignin; CP: crude protein; NDIP: neutral detergent insoluble protein; ADIP: acid detergent insoluble protein; D: drying method; M: maturity stage;

EB: early bud; LB: late bud; F: flowering; LF: late flowering; [a-f] Means within rows with different superscript letters indicate a significant difference at *P* <0.05 ; [w-z] Means within column with different superscript letters indicate a significant difference at *P* <0.05; [A-R] Means within columns and rows (interaction D×M) with different superscript letters indicate a significant difference at *P* <0.05;

Table 3 Effects of drying method on in vitro digestibility and in situ degradability of alfalfa

		Drying method*												P value		
Item	M	TFD	T100	T60	T50	T40	T30	T100+50	TMO	TFREE	Mean	n	SEM	D	M	D×M
In vitro digestibility (g/kg)																
dNDF	EB	460	475	451	453	452	428	470	488	473	461^{w}	27	17.1	**	**	NS
	LB	401	415	362	345	330	370	359	381	387	372^{x}	27				
	F	370	384	343	373	292	357	345	386	371	358^{x}	27				
	LF	311	306	318	290	271	271	327	332	273	300^{y}	27				
	Mean	385^{ab}	395^{a}	369^{abc}	365^{abc}	336^{c}	356^{bc}	375^{ab}	397^{a}	376^{ab}		108				
IVTD	EB	816^{AB}	800^{AB}	811^{AB}	813^{AB}	816^{AB}	796^{AB}	826^{A}	826^{A}	828^{A}	815^{w}	27	7.1	**	**	**
	LB	781^{BC}	733^{DE}	723^{DEF}	716^{DEF}	713^{D-G}	729^{DEF}	736^{DE}	738^{DE}	745^{CD}	735^{x}	27				
	F	731^{DE}	716^{DEF}	693^{FGH}	725^{DEF}	678^{G-J}	701^{EFG}	706^{EFG}	734^{DE}	709^{D-G}	710^{y}	27				
	LF	681^{GHI}	642^{JK}	665^{H-K}	656^{IJK}	646^{IJK}	633^{K}	681^{GHI}	681^{GHI}	661^{H-K}	661^{z}	27				
	Mean	752^{a}	723^{cd}	723^{cd}	727^{bcd}	713^{d}	715^{d}	737^{abc}	745^{ab}	736^{abc}		108				
In situ degradability (g/kg)																
DMD	EB	766^{A}	762^{A}	767^{A}	784^{A}	759^{A}	742^{AB}	768^{A}	756^{A}	744^{AB}	761^{w}	27	9.3	**	**	**
	LB	708^{BC}	676^{CD}	659^{DE}	638^{EFG}	630^{E-H}	650^{DEF}	661^{DE}	638^{EFG}	684^{BCD}	660^{x}	27				
	F	641^{D-G}	622^{E-H}	628^{E-H}	657^{DE}	641^{D-G}	612^{F-I}	615^{FGH}	589^{HIJ}	659^{DE}	629^{y}	27				
	LF	575^{IJ}	530^{K}	613^{F-I}	573^{IJ}	550^{JK}	600^{GHI}	584^{IJ}	555^{JK}	630^{EFG}	579^{z}	27				
	Mean	673^{ab}	647^{cd}	667^{abc}	663^{abc}	645^{cd}	651^{bcd}	657^{abcd}	635^{d}	679^{a}		108				
OMD	EB	746^{AB}	751^{AB}	749^{AB}	769^{A}	737^{AB}	722^{BC}	755^{AB}	737^{AB}	722^{BC}	743^{w}	27	10.0	**	**	**
	LB	684^{C}	661^{CD}	639^{C-F}	615^{D-H}	605^{E-I}	627^{D-G}	643^{CDE}	611^{E-H}	662^{CD}	638^{x}	27				
	F	611^{E-H}	607^{E-I}	603^{E-I}	635^{DEF}	613^{E-H}	583^{G-J}	593^{F-I}	561^{IJK}	637^{DEF}	605^{y}	27				
	LF	546^{JKL}	508^{L}	593^{F-I}	552^{I-L}	524^{KL}	578^{HIJ}	566^{IJK}	533^{JKL}	608^{E-H}	556^{z}	27				
	Mean	647^{ab}	632^{bcd}	646^{ab}	642^{abc}	620^{cd}	628^{bcd}	639^{abc}	610^{d}	657^{a}		108				
CPD	EB	918^{A}	876^{D-G}	912^{AB}	919^{A}	920^{A}	911^{AB}	896^{A-D}	899^{A-D}	911^{AB}	907^{w}	27	5.1	**	**	**
	LB	902^{ABC}	851^{H-K}	871^{E-H}	871^{E-H}	882^{C-F}	893^{B-E}	859^{F-J}	859^{F-J}	883^{C-F}	874^{x}	27				
	F	866^{F-I}	838^{J-M}	856^{G-J}	868^{F-I}	868^{F-I}	855^{G-K}	831^{KLM}	849^{H-K}	866^{F-I}	855^{y}	27				
	LF	844^{I-L}	792^{N}	835^{J-M}	835^{J-M}	837^{J-M}	832^{KLM}	822^{LM}	819^{M}	858^{F-J}	830^{z}	27				
	Mean	882^{a}	839^{d}	869^{b}	873^{ab}	876^{ab}	873^{ab}	852^{c}	856^{c}	880^{a}		108				

**TFD: Freeze-dried; T100: 100 °C for 24 h; T60: 60 °C for 48 h; T50: 50 °C for 48 h; T40: 40 °C for 72 h; T30: 30 °C for 96 h; T100+50: 50 °C for 48 h; TMO: 50 °C for 48 h; TFREE: 50 °C for 48 h; **: *P* <0.01; SEM: standard error of mean NS: not significant; dNDF: in vitro true digestibility of neutral detergent fibre; IVTD: in vitro true digestibility of DM; DMD: dry matter degradability; OMD: organic matter degradability; CPD: crude protein degradability; D: drying method; M: maturity stage; EB: early bud; LB: late bud; F: flowering; LF: late flowering

[a-d] Means within rows with different superscript letters indicate a significant difference at P <0.05

[w-z] Means within column with different superscript letters indicate a significant difference at P <0.05

[A-N] Means within columns and rows (interaction D × M) with different superscript letters indicate a significant difference at P <0.05

enzymatic activity if the enzyme containing organelles, such as lysosomes and mitochondria, were damaged. This explanation may be a reason for the higher in vitro and in situ methods (presented in this study) for the TFREE in comparison with T50. However, the effects of freezing were small, and probably of little practical importance.

As the alfalfa continued to mature, the fibre fractions increased and CP, IVTD, dNDF, DMD, OMD and CPD were significantly (P <0.05) reduced (Tables 2 and 3). The decline in the digestibility and degradability parameters corresponds to the growth of the fibre fractions and ADL amounts during the alfalfa maturation process, which are major factors that limit the digestibility of forages (Van Soest, 1994).

There were no added benefits of the TMO in chemical composition, in vitro digestibility or in situ degradation when compared with the T50. Storing samples in a freezer did not affect the results obtained from the in vitro and in situ methods, compared with the TFD and T50. The FD is considered the best method for preparing samples before analysing their chemical composition and digestibility. However, the current study showed that T50 can yield results that are similar to the FD. Thus, the results of present study may be useful for forage analyses and evaluation.

Acknowledgements
This work was supported by the Ministry of Agriculture of the Czech Republic (Project No. MZERO0715).

Conflict of interest
Authors declare that there is no conflict of interest for this study.

Authors' contributions
Conception and design of study – FJ and PH; harvest and drying of samples – FJ and VK; laboratory analyses and calculations of results – MK; in situ and in vitro analyses – FJ and PK; statistical analyses and draft of manuscript – FJ; All authors commented on early and final versions of the manuscript.

References

Act No. 359/2012 Coll. on protection of animals against maltreatment. In: Collection of Law of Czech Republic. 2012, Item 134/2012, pp. 4746-4805 (in Czech).

Alomar, D., Fuchslocher, R. & Stockebrand, S., 1999. Effects of oven- or freeze-drying on chemical composition and NIR spectra of pasture silage. Anim. Feed Sci. Technol. 80:309-319.

AOAC, 2005. Official Methods of Analysis. 18th edition. AOAC, Gaithersburg, MD, USA.

Cone, J.W., VanGelder, A.H. & Marvin, H.J.P., 1996. Influence of drying method and ageing on chemical and physical properties and in vitro degradation characteristics of grass and maize samples. J. Agric. Sci. 126:7-14.

Damiran, D., DelCurto, T., Bohnert, D.W. & Findholt, S.L., 2008. Comparison of techniques and grinding size to estimate digestibility of forage based ruminant diets. Anim. Feed Sci. Techn. 141:15-35.

Huntington, J.A. & Givens, D.I., 1997. Studies on in situ degradation of feeds in the rumen 3. The effect of freezing forages before and after rumen incubation. Anim. Feed Sci. Techn. 68:131-138.

Licitra, G., Hernandez, T.M. & Van Soest, P.J., 1996. Standardization of procedures for nitrogen fractionation of ruminant feeds. Anim. Feed Sci. Techn. 57:347-358.

Nishino, N., Uchida, S. & Ohshima, M., 1994. Ruminal degradation of alfalfa protein as influenced by sodium hydroxide and heat treatment. Anim. Feed Sci. Techn. 48:131-141.

Pagán, S., Wolfe, R.M., Terrill, T.H. & Muir, J.P., 2009. Effect of drying method and assay methodology on detergent fiber analysis in plants containing condensed tannins. Anim. Feed Sci. Techn. 154:119-124.

Parissi, Z.M., Papachristou, T.G. & Nastis, A.S., 2005. Effect of drying method on estimated nutritive value of browse species using an in vitro gas production technique. Anim. Feed Sci. Techn. 123-124:119-128.

Pelletier, S., Tremblay, G.F., Bertrand, A., Bélanger, G., Castonguay, Y. & Michaud, R., 2010. Drying procedures affect non-structural carbohydrates and other nutritive value attributes in forage samples. Anim. Feed Sci. Techn. 157:139-150.

Purcell, P.J., O´Brien, M., Boland, T.M. & O´Kiely, P., 2011. In vitro rumen methane output of perennial ryegrass samples prepared by freeze drying or thermal drying (40 °C). Anim. Feed Sci. Techn. 166-167:175-182.

SAS Institute Inc. 2002. SAS/STAT User´s Guide: Version 9.1 for window. SAS Institute Inc., Cary, NC, USA.

Van Soest, P.J., 1994. Nutritional ecology of the ruminant. 2nd edition. Cornell University Press, Ithaca, NY, USA.

Van Soest, P.J., Robertson, J.B. & Lewis, B.A., 1991. Methods for dietary fibre, neutral detergent fibre, and non-starch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74:3583-3597.