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Drying procedures affect non-structural carbohydrates and other nutritive value attributes in forage samples

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ABSTRACT

Forage non-structural carbohydrates (NSC) are an important source of energy readily available to rumen microbes. Sampling procedures that quickly reduce plant metabolic activity after cutting and preserve macromolecular structures are required for accurate estimation of forage NSC concentration and other nutritive value attributes. Although several drying procedures exist, their efficiency to reflect composition of fresh samples is unclear. We compared five drying procedures on fresh samples of timothy (*Phleum pratense* L.) and alfalfa (*Medicago sativa* L.): (1) oven-drying at 55 °C for 48 h; (2) high-temperature pretreatment at 100 °C for 1 h followed by oven-drying at 55 °C for 48 h; (3) freezing pretreatment at −20 °C for 1 mo followed by oven-drying at 55 °C for 48 h; (4) microwave pretreatment for 1 min followed by oven-drying at 55 °C for 48 h; and (5) freezing at −20 °C for 1 mo followed by freeze-drying. Starch, sucrose, glucose, fructose, fructans (timothy) or pinitol (alfalfa), N, neutral detergent fibre assayed with a heat-stable amylase (aNDF), acid detergent fibre (ADF), neutral detergent insoluble N (NDIN), and acid detergent insoluble N (ADIN) concentrations, and *in vitro* true digestibility of DM (IVTD) and digestibility of neutral detergent fibre (dNDF) were determined in the spring growth and summer regrowth of two production years. Soluble carbohydrate (SC) concentration was estimated using the sum of sucrose, glucose, fructose, and fructans or pinitol. The NSC concentration was obtained by adding SC and starch. Averaged across forage species and growth periods, NSC concentrations were similar and generally highest ($P<0.001$) in freeze-dried samples (89.8 mg/g DM), the reference procedure, and the microwave-pretreated samples (86.7 mg/g DM); oven-drying at 55 °C resulted in the lowest NSC concentration (57.6 mg/g DM). Values of other nutritive value attributes with the microwave pretreatment were comparable to those of the other drying procedures, except that it tended to increase forage NDIN. The NDIN concentration was lowest ($P<0.001$) in freeze-dried samples (2.1 mg/g DM) and generally highest in microwave-pretreated samples (7.5 mg/g DM). The effect of drying procedure on aNDF, ADF, IVTD, dNDF, and ADIN concentrations varied slightly with forage species and growth period. Principal component analysis (PCA) highlighted the closeness between freeze-drying and microwave pretreatment for the global assessment of the nutritive value of forages, including NSC concentration. Microwave pretreatment should be given greater consideration as an effective and easy-to-use sample preparation procedure for the characterization of the nutritive value of fresh forages.

Abbreviations: ADF, acid detergent fibre; ADIN, acid detergent insoluble N; aNDF, neutral detergent fibre assayed with a heat-stable amylase; DM, dry matter; dNDF, *in vitro* digestibility of neutral detergent fibre; HDP, high degree of polymerization; IVTD, *in vitro* true digestibility of DM; NDIN, neutral detergent insoluble N; NSC, non-structural carbohydrate; PC, principal component; PCA, principal component analysis; SC, soluble carbohydrate.

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1. Introduction

Although accurate estimates of nutritive value are readily obtained from fresh forage samples (Smith, 1981; Valenciaga and Martinez-Machin, 2000; Hove et al., 2003), dried samples offer greater homogeneity and do not need to be chemically analyzed immediately after harvest (Cohen et al., 1995). Several processes occur during the drying of fresh forage samples, including water loss, respiration, loss of volatile organic substances, and protein degradation. While water loss is desirable for preservation of the samples, the other processes can markedly affect the samples' chemical composition and should be minimized (Deinum and Maassen, 1994). For that reason, drying procedures that rapidly inhibit enzyme activity while preserving nutritive value attributes are preferred. To stop metabolic processes, a drying procedure must quickly increase the temperature of the samples or rapidly remove moisture from them (Hofman, 1965; Deinum and Maassen, 1994). However, forage samples should be exposed to a high temperature for only a short time to prevent carbohydrate losses (Heberer et al., 1985).

Freeze-drying is generally recognized as the best method for preserving the labile metabolites, including NSC, that are present in fresh forages (Raguse and Smith, 1965; Heberer et al., 1985). However, freeze-drying requires access to expensive equipment and is difficult to apply to large samples under field conditions. A forced-draft oven is a more convenient approach that is commonly used to dry forage samples but provides conflicting results regarding the preservation of NSC. In perennial ryegrass (*Lolium* spp.), Fulkerson et al. (1998) observed a 14% decrease in the water-soluble carbohydrate concentration in samples dried immediately after harvest in a forced-air oven at 60 or 80 °C for 24 h compared with freeze-dried samples, whereas Smith (1981) determined that grass and legume forage samples that were dried at 100 °C for 1 h or less followed by drying at 70 °C had similar NSC concentrations as freeze-dried samples.

Pretreatment with a microwave oven has also been suggested for forage preparation because it provides very rapid low-temperature predrying (Hofman, 1965). Earlier studies suggested that 1 min of heating in a microwave oven is an efficient method for preparing approximately 300 g fresh forage for water-soluble carbohydrate determination (M. Suzuki, pers. comm.). Pretreatment of samples with a microwave oven for nutritive value determination is not widely performed and, to our knowledge, no attempt has been made to compare this procedure with freeze-drying and oven-drying.

Forage samples are often frozen to preserve them before or after the application of one of the previously mentioned drying methods (Chatterton et al., 1989; Clark et al., 2004; Shewmaker et al., 2006). Skrede (1983) reported that, following thawing of frozen strawberries (*Fragaria ananassa* Duch., var. Senga Sengana), as much as 70% of the sucrose is hydrolyzed by invertases. Park et al. (2002) reported that frozen samples of silages predominantly composed of perennial ryegrass had lower N, neutral detergent fibre, and ADF concentrations than fresh samples.

Most studies have focused on oven-drying for the determination of structural carbohydrates. Increased neutral detergent insoluble nitrogen (NDIN) has been detected by Deinum and Maassen (1994) in the temperature drying range of 30–105 °C, leading to higher cell-wall contents and lower *in vitro* digestibility in several forages and preventing general recommendations about the optimum drying procedure other than freeze-drying. They reported that quick drying of fresh forages at 70 °C was the second best option, because alterations during drying were kept to a minimum (Deinum and Maassen, 1994). Alomar et al. (2003) reported that oven-drying of forage from mixed permanent swards at temperatures between 60 and 100 °C reduces the digestibility of the organic matter content and increases the neutral detergent fibre and insoluble N in neutral detergent fibre compared with freeze-drying. On the other hand, Tilley and Terry (1963) found that forage samples have similar *in vitro* digestibility whether plant material is freeze-dried or oven-dried at 40 or 100 °C.

Very few comparisons have been made in an effort to identify the best procedure for the determination of both structural and non-structural carbohydrates. The objective of this study was to assess the effect of five drying procedures on the concentrations of NSC and other nutritive value attributes in timothy and alfalfa forage samples. We hypothesized that methods involving a rapid rise in temperature or the freezing of forage samples immediately after harvest would best preserve NSC.

2. Materials and methods

Timothy (cv. Champ) and alfalfa (cv. AC Caribou) were sown in 2006 on a St. Pacôme sandy loam soil at St. David-de-Lévis, Lévis, QC, Canada (46°48′N; 71°23′W). Both species made up close to 100% of their respective swards in 2007 and 2008. Harvest dates for both species are shown in Table 1; the dates were chosen to represent a wide range of stages of

Table 1
Date of harvest and stage of development at harvest of the spring growth and summer regrowth of alfalfa and timothy grown in 2007 and 2008 in Lévis, Canada.

	Spring growth		Summer regrowth	
	2007	2008	2007	2008
Date of harvest	21 June	12 June	27 July	26 August
Stage of development				
Timothy	Late heading	Late heading	Heading	Anthesis
Alfalfa	Early flowering	Early bud	Flowering	Seed pod

Table 2
Description of drying treatments applied to forage samples.

Treatment no.	Drying pretreatment	Drying treatment	Treatment abbreviation used in Tables 2 and 3	Treatment description used in Fig. 1B
1	None	Dried at 55 °C for 48 h	55	Oven-drying at 55 °C
2	Dried at 100 °C for 1 h	Dried at 55 °C for 48 h	100	High-temperature pretreatment
3	Frozen in a plastic bag at −20 °C for approximately 1 mo	Dried at 55 °C for 48 h[a]	Free	Freezing pretreatment
4	Heated in a microwave oven[b] for 1 min at maximum intensity to reach approximately 70 °C	Dried at 55 °C for 48 h	MO	Microwave pretreatment
5	Frozen in a plastic bag at −20 °C for approximately 1 mo	Freeze-dried	FD	Freeze-drying

[a] The frozen samples were removed from the plastic bags, put in aluminum pans, and then directly placed in the oven without thawing period.
[b] SAMSUNG, model MS5796W, 1100 W, Samsung Electronics Canada Inc., Mississauga, ON, Canada.

development at harvest. From 1 May to the last harvest date of each production year (27 July in 2007 and 26 August in 2008), the growing degree-day accumulation (base 5 °C) was 963 in 2007 and 1342 in 2008, and total rainfall accumulation was 350 mm in 2007 and 558 mm in 2008. The 30-year average (1971–2000) from 1 May to 31 August is 1417 growing degree-days and 464 mm rainfall (Environment Canada, 2009). The samples were harvested in the morning. About 7 kg of each species was cut on each harvest date with electric pruning shears. The harvested forage was put on ice in five coolers, kept in a refrigerator at 4 °C, and less than 1 h after the cut, it was divided into five replication sets of five sub-samples weighing 250 g each. Each sub-sample in the first set of five was treated using one of the five treatments described in Table 2. The other four replication sets were subsequently treated in the same way. Drying at 55 and 100 °C was done by placing the 250-g forage samples in micro-perforated 28 cm × 51 cm bread plastic bags [Sealed Air (Canada) Inc.-Cryovac, Mississauga, ON, Canada] and then in a forced-air oven (Fisher Scientific Isotemp 750F, chamber capacity of 141.5 L, 45, 5 cm *L* × 45 cm *W* × 67 cm *H*, Fisher Scientific, Ottawa, ON, Canada); two plastic bags containing a forage layer of 6–10 cm were put on each shelf of an oven equipped with five shelves. Dried samples were ground using a Wiley mill (standard model 3; Arthur H. Thomas Co., Philadelphia, PA, USA) to pass through a 1-mm screen.

2.1. Chemical analyses

2.1.1. Carbohydrates

For both species, ground samples were analyzed for starch, sucrose, glucose, fructose, and fructans (timothy) or pinitol (alfalfa). Extraction and determination of NSC were performed according to Pelletier et al. (2009). Briefly, soluble carbohydrates (SC) were extracted from dried samples with a methanol:chloroform:water solution (12:5:3, v/v/v) in a 1:6 (w/v) ratio heated at 65 °C for 30 min, except for fructans from timothy samples that were extracted in hot water (80 °C for 20 min). Carbohydrates were analyzed by high-performance liquid chromatography. Timothy sucrose, glucose, and fructose were separated on a Waters Sugar-Pak column (Waters, Milford, MA, USA) eluted at 80 °C with EDTA (Na^+, Ca^{2+}, 50 mg/L). Alfalfa sucrose, glucose, fructose, and pinitol were separated on a Bio-Rad HPX-87P column (Bio-Rad, Richmond, CA, USA) eluted with deionized water. Elution was performed at a flow rate of 0.5 mL/min for both columns. These carbohydrates were detected on a refractive index detector, and peak identity and quantity were determined by comparison to standards. Low degree of polymerization (LDP) fructans were separated on a Bio-Rad HPX-42A column (Bio-Rad, Richmond, CA, USA), while high degree of polymerization (HDP) fructans were separated with a Shodex KS-804 column (Shodex, Tokyo, Japan). Both columns were eluted isocratically at 25 °C with deionized water at flows of 0.5 and 1.0 mL/min, respectively. Degree of polymerization and abundance were obtained by comparison with standards. The degree of polymerization of LDP fructans was established by comparison with elution time of purified standards from Jerusalem artichoke (*Helianthus tuberosus* L.). The degree of polymerization of HDP fructans was estimated by reference to a standard curve established with seven polymaltotriose pullulan standards (Shodex Standard P-82) ranging from 0.58×10^4 to 85.3×10^4 of molecular weight. The retention time on the Shodex column is function of the log of the molecular weight of pullulan molecules. When an unknown sample containing HDP fructans is eluted on the Shodex column, usually one or two peaks are found with the refractive index detector and each peak corresponds to a given range of degree of polymerization. The concentration of both LDP and HDP fructans is expressed on an equivalent fructose basis.

The non-soluble residues left over after extraction in methanol:chloroform:water were washed twice with methanol and used for starch quantification as a glucose equivalent following enzymatic digestion with amyloglucosidase (Sigma A7255; Sigma–Aldrich Co., St. Louis, MO, USA) and colorimetric detection with the ρ-hydrobenzoic acid hydrazide method of Blakeney and Mutton (1980). The concentration of SC was estimated by determining the sum of sucrose, glucose, fructose, and fructans or pinitol, while the NSC concentration was obtained by adding together the SC and starch values.

2.1.2. Other nutritive value attributes

Nitrogen was extracted using a method adapted from Isaac and Johnson (1976). Samples (100 mg each) were digested for 60 min at 380 °C in a 1.5-mL mixture of selenious and sulphuric acids (1:42) plus 2 mL 30% H_2O_2. After cooling, the mixture was diluted to 75 mL with deionized water. Nitrogen was then determined on a QuikChem 8000 Lachat autoanalyzer with method 15-107-06-2-E (Zellweger Analytics, Inc., Lachat Instruments, Milwaukee, WI, USA).

The concentration of neutral detergent fibre assayed with a heat-stable amylase (aNDF) was determined according to Mertens (2002), and heat-stable alpha amylase and sodium sulphite were both added during extraction. Acid detergent fibre (ADF) was determined according to method 973.18 of the Association of Official Analytical Chemists (AOAC, 1990). All extractions were done on an ANKOM220 Fibre Analyzer (ANKOM Technology 05/03, Macedon, NY, USA) using F57 filter bags (25-μm porosity), and aNDF and ADF values were expressed inclusive of residual ash. Neutral detergent insoluble N (NDIN) and acid detergent insoluble N (ADIN) were determined according to the method of Licitra et al. (1996), in which amylase and sodium sulphite are omitted during neutral detergent fibre determination. Residues of neutral detergent fibre and ADF were kept in a desiccator before determination of NDIN and ADIN. Then, 0.1 g of each residue sample was weighed in a digestion tube for N extraction and determination as previously described.

The *in vitro* true digestibility of DM (IVTD) was measured using the method of Van Soest et al. (1966) based on a 48-h incubation with buffered rumen fluid followed by an aNDF determination of the postdigestion residues. The rumen fluid incubation was performed with ANKOM F57 filter bags and an ANKOM Daisy II incubator, using the batch incubation procedures outlined by ANKOM Technology Corp. (Macedon, NY, USA) and described by Wilman and Adesogan (2000). Rumen fluid was obtained from a lactating, ruminally fistulated dairy cow that was offered a diet of good quality silages [35% grass, 24% corn (*Zea mays* L.), and 41% hay in 2007; 48% grass, 36% corn, and 16% hay in 2008], corn grain, and a concentrate mix. The diet was formulated to meet the nutritional requirements of a lactating cow expected to produce 10,300 kg milk per year. The IVTD (g/kg DM) and the *in vitro* true digestibility of neutral detergent fibre (dNDF; g/kg aNDF) were calculated as follows:

$$\text{IVTD} = \left[1 - \left(\frac{\text{postdigestion dry weight following aNDF wash}}{\text{predigestion dry weight}}\right)\right] \times 1000$$

$$\text{dNDF} = \left[1 - \left(\frac{\text{postdigestion dry weight follwing aNDF wash}}{\text{predigestion dry weight of aNDF}}\right)\right] \times 1000$$

All laboratory analyses of nutritive value attributes were done in duplicate on each forage sample.

2.2. Statistical analyses

Data normality was verified using the Shapiro–Wilk statistic, and the variance homogeneity was verified visually with graphics of the residuals (SAS Institute, 1999). Raw data were transformed (logarithmic transformation) when deemed appropriate. An analysis of variance using the MIXED procedure of SAS (Littell et al., 1996) was first conducted with species, growth periods, and drying methods as fixed effects. Because of the interactions ($P<0.05$) between drying methods and the other two factors, data for each forage species and growth period (n = two production years × five drying methods × five replicates = 50) were analyzed separately as a randomized complete block with five replicates using the MIXED procedure of SAS (Tables 3 and 4). Replicates and production years were considered to be random effects. Statistical significance was postulated at $P\leq0.05$. Least squares means are reported. Comparisons of least squares means were carried out using the predicted difference (PDIFF) option of SAS with the Tukey–Kramer adjustment. Principal component analysis (PCA) was used to understand relationships among nutritive value attributes and treatments and was performed on the least squares means using the correlation matrix method (SAS Institute, 1999) to give equal weight to all attributes.

3. Results

3.1. Non-structural carbohydrates

Drying procedures affected ($P<0.001$) the concentrations of NSC and its components in timothy and alfalfa spring growth and summer regrowth, with the exception of pinitol (Table 3). Variations in carbohydrate concentrations among drying procedures were generally similar in both forage species and growth periods, but the intensity of these variations varied; therefore, the results reported hereafter are average values across forage species and growth periods unless otherwise mentioned. The NSC and SC concentrations were highest in the freeze-dried (89.8 mg/g DM) and microwave-pretreated (86.7 mg/g DM) samples and did not differ between these two drying procedures.

The freeze-dried samples generally had high concentrations of glucose (22.0 mg/g DM) and fructose (13.2 mg/g DM) and an intermediate concentration of sucrose (16.9 mg/g DM) compared to the other drying procedures. There were no LDP fructans in any timothy samples of the current study (data not shown). The concentration of HDP fructans, detected only in timothy summer regrowth, was not different in the freeze-dried samples than in the microwave-pretreated samples.

The microwave pretreatment resulted in a higher sucrose concentration (25.3 mg/g DM; averaged across growth periods and forage species) and a numerically lower HDP concentration (44.8 mg/g DM) compared with freeze-drying (Table 3). In

Table 3
Carbohydrate composition of timothy and alfalfa spring growth and summer regrowth dried using one of five procedures: drying in a forced-air oven dryer at 55 °C for 48 h (55); pretreatment in a forced-air oven dryer at 100 °C for 1 h then at 55 °C for 48 h (100); pretreatment by freezing then drying in a forced-air oven dryer at 55 °C for 48 h (Free); pretreatment in a microwave oven for 1 min then drying in a forced-air oven dryer at 55 °C for 48 h (MO); or freeze-drying (FD). Forages were grown in Lévis, Canada, and values are averages over two growing seasons, 2007 and 2008.

Species	Growth period	Drying method	NSC[a] (mg/g DM[b])	SC[c] (mg/g DM[b])	Starch (mg/g DM[b])	Sucrose (mg/g DM[b])	Glucose[d] (mg/g DM[b])	Fructose (mg/g DM[b])	HDP fructans (mg/g DM[b])	Pinitol (mg/g DM[b])
Timothy	Spring growth	55	38.6^{a}	33.1^{a}	5.5^{a}	24.8^{c}	5.1^{a}	2.9^{a}	0.3	0.0
		100	58.0^{b}	50.9^{b}	7.1^{b}	19.1^{bc}	20.3^{b}	11.5^{b}	0.0	0.0
		Free	64.3^{bc}	57.3^{bc}	7.0^{b}	2.5^{a}	31.0^{c}	23.8^{d}	0.0	0.0
		MO	64.6^{bc}	56.9^{bc}	7.7^{b}	25.6^{c}	21.7^{b}	9.0^{b}	0.6	0.0
		FD	71.2^{c}	64.3^{c}	6.9^{b}	14.6^{b}	31.2^{c}	16.5^{c}	2.0	0.0
		P value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		
	Summer regrowth	55	83.8^{a}	77.1^{a}	6.7^{a}	32.5^{c}	6.3^{a}	5.4^{a}	32.9^{a}	0.0
		100	88.1^{a}	80.3^{a}	7.8^{ab}	22.6^{b}	12.1^{b}	9.4^{b}	36.2^{a}	0.0
		Free	94.5^{ab}	87.3^{ab}	7.2^{ab}	4.5^{a}	24.2^{d}	24.0^{c}	34.6^{a}	0.0
		MO	105.3^{bc}	97.2^{b}	8.1^{ab}	31.4^{c}	12.9^{b}	8.1^{ab}	44.8^{ab}	0.0
		FD	109.2^{c}	101.0^{b}	8.2^{b}	20.7^{b}	17.5^{c}	10.5^{b}	52.3^{b}	0.0
		P value	<0.001	<0.001	0.018	<0.001	<0.001	<0.001	<0.001	
Alfalfa	Spring growth	55	48.6^{a}	39.6^{a}	9.0^{a}	14.2^{b}	7.4^{a}	2.6^{a}	0.0	15.0^{a}
		100	80.0^{b}	53.7^{b}	26.3^{b}	13.0^{b}	16.8^{b}	10.9^{b}	0.0	16.5^{a}
		Free	85.2^{bc}	56.0^{bc}	29.2^{b}	3.9^{a}	22.3^{bc}	18.2^{c}	0.0	16.9^{a}
		MO	87.6^{bc}	64.0^{cd}	23.6^{b}	19.9^{c}	19.1^{bc}	11.2^{b}	0.0	17.9^{a}
		FD	93.5^{c}	66.7^{d}	26.8^{b}	13.1^{b}	23.2^{c}	15.7^{c}	0.0	17.7^{a}
		P value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		NS[e]
	Summer regrowth	55	59.6^{a}	45.2^{a}	14.4^{a}	23.4^{c}	9.2^{a}	3.0^{a}	0.0	9.5^{a}
		100	73.1^{b}	47.6^{a}	25.5^{bc}	14.9^{b}	12.6^{ab}	8.7^{b}	0.0	11.3^{a}
		Free	71.5^{ab}	51.4^{ab}	20.1^{ab}	4.5^{a}	18.3^{c}	16.9^{c}	0.0	11.6^{a}
		MO	89.5^{c}	60.4^{c}	29.1^{c}	24.1^{c}	15.6^{bc}	9.5^{b}	0.0	11.3^{a}
		FD	85.2^{c}	56.6^{bc}	28.6^{c}	19.0^{bc}	15.9^{bc}	9.9^{b}	0.0	11.9^{a}
		P value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		NS

[a,b,c,d] Different letters within a column, growth period, and forage species indicate significant differences ($P<0.05$).

[a] NSC = SC + starch. NSC, non-structural carbohydrates; SC, soluble carbohydrates.

[b] Dry matter.

[c] Timothy SC = sucrose + glucose + fructose + HDP fructans; alfalfa SC = sucrose + glucose + fructose + pinitol. HDP, high degree of polymerization.

[d] When the statistical analysis was performed on raw data, least squares means are reported; when the statistical analysis was performed on transformed data (alfalfa glucose), detransformed least squares means are reported.

[e] Not significant.

Table 4
Nitrogen and fibre composition and digestibility of timothy and alfalfa spring growth and summer regrowth dried using one of five procedures: drying in a forced-air oven dryer at 55 °C for 48 h (55); pretreatment in a forced-air oven dryer at 100 °C for 1 h then at 55 °C for 48 h (100); pretreatment by freezing then drying in a forced-air oven dryer at 55 °C for 48 h (Free); pretreatment in a microwave oven for 1 min then drying in a forced-air oven dryer at 55 °C for 48 h (MO); or freeze-drying (FD). Forages were grown in Lévis, Canada, and values are averages over two growing seasons, 2007 and 2008.

Species	Growth period	Drying method	N (mg/g DM[a])	ADF[b] (mg/g DM[a])	aNDF[c] (mg/g DM[a])	NDIN[d] (mg/g DM[a])	ADIN[e] (mg/g DM[a])	IVTD[f] (mg/g DM[a])	dNDF[g] (mg/g aNDF[c])
Timothy	Spring growth	55	16.7[a]	377[c]	657[bc]	4.4[b]	0.57[ab]	744[a]	611[a]
		100	16.0[a]	376[bc]	654[bc]	6.6[c]	0.78[bc]	757[a]	630[a]
		Free	16.0[a]	376[bc]	664[c]	7.0[c]	0.91[c]	761[a]	641[ab]
		MO	16.0[a]	365[ab]	644[b]	6.8[c]	0.65[b]	783[b]	665[bc]
		FD	15.6[a]	359[a]	620[a]	1.7[a]	0.43[a]	797[b]	675[c]
		P value	NS[h]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	Summer regrowth	55	17.5[a]	332[ab]	569[b]	5.1[b]	0.68[a]	832[ab]	705[ab]
		100	17.4[a]	330[ab]	579[bc]	7.2[c]	0.93[b]	845[b]	732[b]
		Free	17.0[a]	334[b]	586[c]	7.9[c]	1.16[c]	843[b]	732[b]
		MO	16.5[a]	327[ab]	566[b]	7.1[c]	0.85[ab]	818[a]	679[a]
		FD	16.7[a]	323[a]	532[a]	2.4[a]	0.69[a]	840[b]	699[ab]
		P value	NS	0.025	<0.001	<0.001	<0.001	0.006	<0.001
Alfalfa	Spring growth	55	34.3[b]	295[b]	353[b]	3.5[b]	1.32[b]	828[a]	519[a]
		100	33.6[ab]	279[ab]	332[ab]	6.6[c]	1.32[b]	842[a]	535[a]
		Free	33.2[ab]	273[ab]	332[ab]	4.0[b]	1.01[a]	841[a]	539[a]
		MO	33.8[ab]	267[a]	325[a]	8.6[d]	1.01[a]	847[a]	545[a]
		FD	31.8[a]	278[ab]	337[ab]	1.9[a]	1.18[ab]	844[a]	546[a]
		P value	0.015	0.023	0.034	<0.001	<0.001	NS	NS
	Summer regrowth	55	30.8[a]	323[a]	385[a]	3.1[a]	1.50[a]	791[a]	458[a]
		100	32.2[a]	307[a]	359[a]	6.2[c]	1.42[a]	813[ab]	481[a]
		Free	31.7[a]	313[a]	370[a]	4.8[b]	1.28[a]	804[ab]	473[a]
		MO	30.6[a]	296[a]	361[a]	7.6[d]	1.26[a]	815[ab]	489[a]
		FD	30.1[a]	307[a]	359[a]	2.5[a]	1.43[a]	818[b]	494[a]
		P value	NS	NS	NS	<0.001	NS	0.030	NS

[a,b,c,d]Different letters within a column, growth period, and forage species indicate significant differences ($P<0.05$).

[a] Dry matter.
[b] Acid detergent fibre.
[c] Neutral detergent fibre assayed with a heat-stable amylase.
[d] Neutral detergent insoluble N.
[e] Acid detergent insoluble N.
[f] *In vitro* true digestibility.
[g] Digestible neutral detergent fibre.
[h] Not significant.

timothy, a lower glucose concentration was observed in the microwave-pretreated samples (17.3 mg/g DM; averaged across growth periods) compared with the freeze-dried samples (24.4 mg/g DM), but in alfalfa, the glucose concentration was similar in both the microwave-pretreated samples and the freeze-dried samples. In spring growth, the fructose concentration was lower in the microwave-pretreated samples (10.1 mg/g DM; averaged across species) than in the freeze-dried samples (16.1 mg/g DM; averaged across species), but in summer regrowth, both drying procedures resulted in similar fructose concentrations in the two forage species.

The samples that underwent the freezing and high-temperature (100 °C) pretreatments yielded intermediate NSC and SC concentrations; those two procedures were not different from each other (Table 3). The NSC and SC concentrations were numerically higher in the freeze-dried samples compared with the freezing-pretreated samples. The freezing-pretreated samples had high concentrations of glucose (24.0 mg/g DM) and fructose (20.7 mg/g DM) and the lowest sucrose concentration (3.9 mg/g DM) compared with the other four drying procedures. The HDP fructans in the freezing-pretreated samples were lower than those in the freeze-dried samples but were not different from those in both the oven-dried samples and the microwave-pretreated samples.

Drying at 55 °C without pretreatment resulted in lower ($P<0.05$) concentrations of NSC (57.6 mg/g DM) and SC (48.7 mg/g DM) than those found with freeze-drying and microwave pretreatment (Table 3). Moreover, the samples oven-dried at 55 °C had the lowest concentrations of glucose (7.0 mg/g DM), fructose (3.5 mg/g DM), and starch (8.9 mg/g DM) (Table 3), but the sucrose concentration in the samples oven-dried at 55 °C was in the range of the other drying procedures.

The high-temperature-pretreated samples had higher concentrations of NSC (74.8 mg/g DM) and SC (58.1 mg/g DM) compared with the samples dried at 55 °C (Table 3). A high-temperature pretreatment allows faster heating of the samples and shortens the time needed to reach dryness, which may reduce metabolic losses of carbohydrates.

3.2. Other nutritive value attributes

The freeze-dried samples tended to have lower concentrations of ADF (317 mg/g DM) and aNDF (462 mg/g DM) than the samples treated with the other drying procedures (323 mg ADF/g DM, 484 mg aNDF/g DM) (Table 4); this effect was more important for timothy than alfalfa. The freezing pretreatment tended to increase the ADF and aNDF concentrations, but only in the timothy samples (Table 4).

The N concentration was not affected by the drying procedures except for the spring growth of alfalfa, where oven-drying at 55 °C slightly increased the N concentration compared with freeze-drying (Table 4).

For both timothy and alfalfa, the NDIN concentration in the freeze-dried samples (2.1 mg/g DM) was markedly lower than that found with the other four drying procedures (6.0 mg/g DM) (Table 4). On average across growth periods, the freezing-pretreated timothy samples tended to have a higher NDIN concentration (7.5 mg/g DM) than the corresponding alfalfa samples (4.4 mg/g DM). On average across forage species and growth periods, the NDIN concentration tended to be higher in the microwave-pretreated samples (7.5 mg/g DM) than in the samples subjected to the other four drying procedures (4.7 mg/g DM), including the high-temperature pretreatment at 100 °C.

The ADIN concentration was less affected by drying procedures than the NDIN concentration (Table 4). On average across growth periods, the timothy ADIN concentration was lowest in the freeze-dried samples (0.56 mg/g DM) and highest in the freezing-pretreated samples (1.04 mg/g DM). The ADIN concentration in alfalfa spring growth was higher in the samples oven-dried at 55 °C without pretreatment or pretreated at high temperature (100 °C) (average of 1.32 mg/g DM) and lower in the freezing- and microwave-pretreated samples (average of 1.01 mg/g DM). In summer regrowth, the ADIN concentration in alfalfa was not affected by drying procedures.

Drying procedures affected ($P<0.01$) the IVTD and dNDF of timothy, but their effect on the IVTD and dNDF of alfalfa was not always significant (Table 4). Overall, the IVTD and dNDF tended to be higher in the freeze-dried and microwave-pretreated samples than in the samples subjected to the other drying procedures; oven-drying at 55 °C resulted in the lowest IVTD and dNDF except for the timothy summer regrowth. However, variations in digestibility in the freeze-dried and microwave-pretreated samples compared to the samples subjected to the other three drying procedures remained low (from −11 to +36 mg/g DM for IVTD and from −34 to +43 mg/g aNDF for dNDF). The IVTD and dNDF values of the freezing-pretreated samples were intermediate among all the drying procedures.

3.3. Relationships among attributes

A principal component analysis was performed to assess how variations in the attributes of forage nutritive value (Fig. 1A) are spatially related to the drying procedures (Fig. 1B). Because variations in carbohydrate concentrations among the drying procedures were generally similar in forage species and growth periods, PCA was conducted on the average values of two species (timothy and alfalfa) and two growth periods (spring growth and summer regrowth). The contribution of each attribute to a principal component axis can be seen from its loadings (Fig. 1B).

There is a contrasted distribution of the drying procedures along the first two principal components that collectively explains 85% of the variability among the nutritive value attributes. Microwave pretreatment and freeze-drying are in close proximity, indicating that these two methods provide highly similar nutritive value estimates. Conversely, oven-drying at 55 °C is opposed to both freeze-drying and microwave pretreatment (Fig. 1B). The important contrast between these procedures is explained by the lower NSC concentrations in the samples oven-dried at 55 °C compared to the freeze-dried

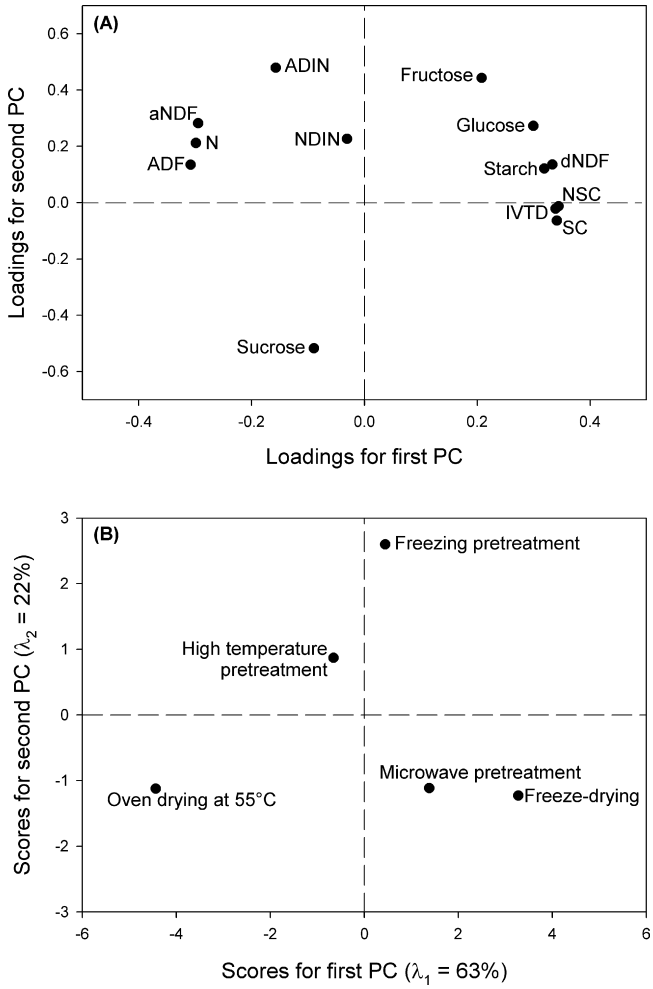

Fig. 1. Diagrams of the first two principal component (PC) loadings (A, attributes) and scores (B, drying procedures). Values are based on the average of two species (timothy and alfalfa), two growth periods (spring growth and summer regrowth) and two production years (2007 and 2008).

and microwave-pretreated samples, as indicated by the large positive loadings for NSC on the axis of the first component (Fig. 1A). The second component of the PCA explains 22% of the total variability. It is driven by the freezing pretreatment (Fig. 1B) and defines a contrast between the sucrose concentration and both the glucose and fructose concentrations (Fig. 1A).

4. Discussion

4.1. Non-structural carbohydrates

The carbohydrate concentrations in timothy and alfalfa observed in the present study (Table 3) are similar to the values reported in the literature (Tremblay et al., 2005; Burns et al., 2007; Bertrand et al., 2008). Moreover, the much higher concentration of HDP fructans in summer regrowth of timothy compared to spring growth was also reported in Pelletier et al. (2009).

The high NSC and SC concentrations in the freeze-dried samples compared to samples subjected to the other drying procedures was expected, because freeze-drying generally provides results that are closest to those from fresh material (Smith, 1981). Deinum and Maassen (1994) observed higher water-soluble carbohydrate concentrations (average of +59%) in freeze-dried radish (*Rhaphanus sativus* L.), alfalfa, ryegrass (*Lolium multiflorum* Lam.), and corn silage compared with samples oven-dried at temperatures ranging from 30–105 °C.

Consistently lower sucrose concentration in freeze-dried samples than in microwave-pretreated samples could be due to invertases that remained active during freeze-drying leading to interconversions between carbohydrate fractions (Raguse and Smith, 1965).

The similar results in NSC and SC concentrations between freezing and high-temperature pretreatments were also reported by Deinum and Maassen (1994). These authors reported no significant effect on water-soluble carbohydrate con-

centrations when samples of four forage species were frozen at −20 °C before drying at 70 °C compared with drying at 70 °C alone. The high concentrations of glucose and fructose in combination with the lowest sucrose concentration in the freezing-pretreated samples compared with the other four drying procedures suggest strong invertase activity, likely occurring when frozen samples are transferred to 55 °C. Moreover, the slightly lower NSC and SC concentrations in the freezing-pretreated samples compared with the freeze-dried samples could be due to respiratory losses during thawing (Griggs et al., 1999), enzymatic conversion (Skrede, 1983), or metabolite leaching.

The lower NSC and SC concentrations found in samples dried at 55 °C without pretreatment compared to those found with freeze-drying and microwave pretreatment suggests that drying at temperatures that do not denature proteins accelerates enzymatic conversions and respiratory losses (Smith, 1973). This is supported by the lowest concentrations of glucose, fructose, and starch in samples oven-dried at 55 °C compared to samples dried with the other drying procedures. Considering that the sucrose concentration in the samples oven-dried at 55 °C was in the range of the other drying procedures, it is likely that monosaccharides were lost through metabolic processes rather than converted to sucrose or starch.

The higher concentrations of NSC and SC in the high-temperature-pretreated samples compared with the samples dried at 55 °C are due to higher glucose, fructose, and starch concentrations. A high-temperature pretreatment allows faster heating of the samples and shortens the time needed to reach dryness, which may reduce metabolic losses of carbohydrates. Deinum and Maassen (1994) also found that water-soluble carbohydrate concentrations increased in four forage species with increasing oven-drying temperatures up to 105 °C, and, similarly to the current study, that the concentrations were lower than those in freeze-dried samples. Forage NSC can be lost when oven-drying at a high temperature is not done in conditions allowing the fast removal of moisture (Smith, 1973).

In the present study, pinitol in alfalfa was not affected by the drying procedures. Pinitol, a carbocyclic inositol (C6 cyclitols), is considered a sugar alcohol that resembles carbohydrates in its properties and is classified as such by Robinson (1993). Pinitol is found in several legume species (Smith and Phillips, 1980) and accumulates when plants are submitted to environmental stresses such as drought (Liu et al., 2008) and cold (Bertrand et al., 2007). Pinitol is known to protect plant enzymes from heat inactivation (Laurie and Stewart, 1990; Guo and Oosterhuis, 1995). Pinitol was included in our quantification of soluble sugars because it is found at concentrations higher or comparable to sucrose (Bertrand et al., 2007; Brito et al., 2008, 2009; Streeter et al., 2001; McManus et al., 2000). Furthermore, Lowry and Kennedy (1994) concluded that its value as an energy source for the ruminant can be equated with soluble sugars.

4.2. Other nutritive value attributes

The trend in lower fibre concentration in the freeze-dried samples compared to samples treated with the other drying procedures was also observed in previous studies. Decreases in the neutral detergent fibre concentration in freeze-dried samples compared with samples oven-dried at temperatures above 45 °C are reported for grass silages, mixed grass swards, and shrub and tree species of the Mediterranean area (Parissi et al., 2005). However, inconsistent effects of drying procedures are reported for ADF concentration (Alomar et al., 1999, 2003; Palmer et al., 2000). The greater effect of freeze-drying on fibre concentrations of timothy compared to alfalfa may be related to the higher fibre concentrations in timothy than in alfalfa.

The limited effect of the freezing pretreatment on forage fibre concentrations is also reported by Park et al. (2002) on the near-infrared reflectance spectroscopy prediction of grass neutral detergent fibre and ADF concentrations and by Alomar et al. (2003) who observed that the ADF and neutral detergent fibre concentrations in mixed grasses were not affected when the samples were frozen, whether by liquid N or in a conventional freezer, prior to freeze-drying or oven-drying. The ADF can be increased by the formation of Maillard reaction products, which occurred when forages are dried at high temperatures (Deinum and Maassen, 1994). These indigestible products are condensates of carbohydrates and amino acids that cause a decrease in carbohydrate and true protein concentrations and an increase in cell-wall content compared with fresh material. Nevertheless, to prevent the formation of Maillard products, it has been suggested that samples be oven-dried at a high temperature until dryness (10% moisture) and that drying be completed at 70 °C (Smith, 1973; Alomar et al., 2003). Fast drying is essential to prevent the formation of Maillard products (Deinum and Maassen, 1994), and forage samples dry faster when they are spread out and held in porous containers during drying.

Similar to our results of N concentration, Heberer et al. (1985) observed no differences in reduced N and nitrate N when samples of corn, soybean (*Glycine max* [L.] Merr.), wheat (*Triticum aestivum* L.), and sunflower (*Helianthus annuus* L.) were oven-dried at temperatures up to 100 °C compared with freeze-drying.

The lower NDIN concentration of the freeze-dried samples compared with that of the other four drying procedures is to be expected, as freeze-drying was primarily developed for the determination of undenatured protein (Smith, 1981). Alomar et al. (2003) also observed an increase in the NDIN concentration (+247%) when mixed swards were oven-dried at 60 °C for 48 h compared with freeze-drying.

The trend for a higher NDIN concentration in the microwave-pretreated samples might indicate that the temperature of the forage samples in the microwave oven was higher than the targeted 70 °C or that the steep increase in temperature within the forage samples combined with high moisture helped to promote protein aggregation. There are also reports that microwave radiation at high intensity may induce non-thermal rearrangements of protein structure (Porcelli et al., 1997) and cause protein denaturation (Bohr and Bohr, 2000), although there is conflicting evidence in that regard (Shazman et al.,

2007). It is apparent from the comparison of the drying methods that increases in the NDIN concentration mainly resulted from thermal effects. In the present study, the NDIN concentration was higher in the samples pre-exposed for 1 h to a high temperature (100 °C) as compared to those oven-dried at 55 °C only. An increase in the NDIN concentration with an increase in the oven-drying temperature was also reported with *Calliandra calothyrsus* shrubs, radish, alfalfa, ryegrass, and corn silage (Deinum and Maassen, 1994; Palmer et al., 2000). Those studies indicate that plant characteristics (age, species, and fraction) and growth conditions (N fertilization, temperature, and light intensity) affect N solubility in oven-drying at temperatures over 70 °C and cause errors in the true measurement of plant cell-wall content.

Few studies report on variations of ADIN concentration with drying procedures. In our study, the ADIN concentration divergently varied with drying procedures depending on forage species and growth periods. Hove et al. (2003) observed that shrub legumes had a higher ADIN concentration when dried at 55 °C compared to fresh material.

According to the Cornell Net Carbohydrate and Protein System, the forage NDIN concentration includes three fractions of true protein (B_1, B_2, and B_3) and a C fraction composed of heat-damaged protein and N associated with lignin, while the ADIN concentration includes the C fraction only (Licitra et al., 1996). The smaller effect of the microwave pretreatment on the ADIN concentration compared with the NDIN concentration suggests that this procedure affected the B fractions more than the C fraction.

In the present study, the effects of drying procedures on the IVTD and dNDF were inconsistent among forage species and growth periods and generally remained low. Alomar et al. (2003) reported that the *in vitro* digestible OM content of mixed grass swards was not affected when the samples were frozen, whether by liquid N or in a conventional freezer, prior to freeze-drying or oven-drying. Park et al. (2002) observed a limited effect of freezing and thawing on the NIRS prediction of grass digestible OM in the DM.

4.3. Relationships among attributes

The efficiency in rapidly stopping enzyme activity after harvest is reflected by the first component of the PCA analysis. It is likely that the sample temperatures increased faster with the microwave pretreatment than with oven-drying procedures, leading to a better preservation of carbohydrates with the microwave pretreatment. On the other hand, oven-drying at 55 °C alone is the slowest procedure in terms of increasing sample temperature, leading to important carbohydrate losses. The position of the freezing pretreatment along the first component also reflects the relative efficiency of this procedure for the conservation of NSC. In this case, enzyme activity was stopped by the freezing temperature instead of heat inactivation or tissue dehydration. The position of this treatment along the second component, however, is explained by the important interconversion of sucrose into glucose and fructose observed in the freezing-pretreated samples. These changes likely occurred during thawing of the freezing pretreatment, because no important interconversion occurred in the freeze-dried samples that were also kept frozen at −20 °C. It is therefore likely that enzyme activity resumes during thawing in the dryer at 55 °C when the sample temperature has not yet increased enough to affect enzyme activity. The freeze-drying process keeps the samples cold, and water is removed by ice sublimation, preventing the activation of enzymes. The samples are returned to room temperature only when completely dry.

The negative correlation often observed between the NSC and fibre concentrations is also clearly illustrated by the first principal component (Fig. 1A), confirming that drying procedures that provide better preservation of NSC will result in lower concentrations of structural carbohydrates (ADF and aNDF). Conversely, the commonly used practice of oven-drying forage samples at 55 °C is likely to result in an overestimation of the ADF and aNDF concentrations. Some variations in fibre and N fractions may be explained only as a result of enrichments or losses of other components.

Our results show that the microwave pretreatment reflects the fresh material composition in terms of digestibility, fibre concentrations, and NSC and its components. The wide variations observed for some nutritive value parameters (*e.g.* NSC, N, ADF, aNDF, and dNDF) between growth periods or forage species did not affect the conclusions of our study. Our findings indicate that forage nutritive value attribute analyses following a microwave pretreatment provide reliable assessments of nutritive value regardless of forage material (*e.g.* species and stage of development) or growth conditions (*e.g.* temperatures and rainfall).

5. Conclusions

For fresh timothy and alfalfa forage samples, microwave pretreatment followed by oven-drying at 55 °C for 48 h resulted in estimates of NSC concentration that were very similar to the estimates determined after freeze-drying, the reference procedure. Oven-drying at 55 °C without microwave pretreatment must be avoided when NSC values are to be determined on dried forage samples because of the important decrease in the NSC concentration compared with freeze-drying and microwave pretreatment. The results of other nutritive value attributes with the microwave pretreatment were comparable to those found with the freeze-drying procedure, except that microwave pretreatment tended to increase forage NDIN. Microwave pretreatment is an easy and fast procedure that can be applied to a wide range of forage species and growth conditions, and it represents an alternative to freeze-drying when that procedure is not feasible.

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