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Effect of freezing and grinding method on near-infrared reflectance (NIR) spectra variation and chemical composition of fresh silage

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Abstract

In order to evaluate possible effects of rapid freezing and grinding methods on near-infrared reflectance (NIR) spectra variability and chemical composition, samples from 18 pasture silages, previously pre-homogenized, were frozen either with liquid nitrogen (LN) or dry ice (DI) and, subsequently, ground with a Tecator® homogenizer (Tec) or a Moulinex® food processor (Mou). NIR spectra were taken (monochromator NIRSystems 6500) on four fresh subsamples per silage and root mean squares (RMS) computed on derivatized spectra (software NIRS 3, ISI, 1992) as a means of estimating spectra dissimilarities among subsamples. Principal components were computed and samples plotted according to the first three scores to visualize potential effects of treatments. Seven silages from the four treatments plus a control (pre-homogenized only) were freeze-dried and analyzed for toluene dry matter (DM_{Tol}), crude protein (CP) and acid detergent fiber (ADF). Average RMS value for silages was 423.97 and no effect of freezing or grinding method, nor any interaction between them, was found ($P > 0.05$), although DI-Mou and DI-Tec treatments showed lower (402.8) and higher (459.4) mean RMS values, respectively. Plotting of samples according to the main principal component scores of spectra did not show any effect of treatments, as samples tended to cluster by silage instead as by treatment. Chemical composition of treated, and control samples, was not different ($P > 0.05$). On average, composition was as follows: DM_{Tol}, 269.2 g kg^{-1}; CP and ADF (oven DM basis), 126.0 and 403.2 g kg^{-1} DM, respectively. It is concluded that any sample handling method could be selected for calibration and subsequent routine analysis, but if a method is chosen, it is suggested to consistently adhere to that method.

Keywords: Freezing; Grinding; NIR; Near-infrared reflectance; Silage; Sample handling; Chemical composition

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1. Introduction

Near-infrared reflectance (NIR) spectroscopy is a non-destructive and potentially precise technique for rapid and multiple prediction of nutritionally relevant traits of many feeds including silages. Although prediction of composition of dried samples has been carried out for a number of years, the analysis of undried silages by NIR spectroscopy has the major advantages of reducing time of sample preparation and of reporting the results (Kennedy et al., 1996; De la Roza et al., 1996; Shenk and Westerhaus, 1995). Another reason for the case of direct analysis of wet silage samples is that oven-drying, a standard laboratory method, can alter chemical composition by volatilisation of organic compounds, and by creating Maillard products with the effects of increasing fractions, such as cell walls or insoluble nitrogen (Deinum and Maassen, 1993). In developing a calibration, the goal of sample presentation when collecting a spectrum, is to obtain optical data that represent the chemical composition of the sample submitted for reference analysis. For a coarse material, such as silage, multiple scans and subsampling are suggested to compensate for its uneven nature and, at the same time, an estimate of dissimilarity among subsamples is recommended in order to maintain variation under acceptable limits (ISI, 1992). On the other hand, particle size, among other factors, is known to affect diffuse reflectance as it is a major factor responsible for light scattering (Murray, 1993). One way to reduce particle size and variation, to speed processing and to facilitate handling of fresh samples, could be the rapid grinding of previously frozen samples. Freezing with liquid nitrogen has been adopted in some laboratories as a means of achieving rapid mechanical shattering with a minimum of water losses, prior to the direct determination of gross energy in fresh samples of forages (Moss et al., 1992; Givens et al., 1989). Rapid pre-processing is a relevant issue for routine NIR spectral analysis and as calibration and subsequent routine prediction should be performed in a consistent way, a proper method of sample preparation should be devised prior to taking spectra. The objective of this work was to evaluate the potential effects of two methods of rapid freezing and two methods of grinding, on some spectral features and chemical composition of fresh pasture silages. As this work deals with a preliminary goal of selecting an acceptable sample-handling method, no calibration was developed at this stage.

2. Material and methods

The study was performed with 18 samples of silages made from grass and mixed permanent pastures, mainly comprising grasses from the genera *Agrostis*, *Lolium*, *Bromus* and *Holcus*, from the humid, temperate southern Chile (IX and X Regions). Not all samples could be processed as they were collected, therefore they were all frozen (−15°C) as soon as received at the laboratory, and conserved as such until used. Although some effects of prior freezing on spectral features through cell-wall disruption could be expected, freezing of silage samples is a normal procedure in our laboratory to put them on queue for analysis, at peak working periods. Besides, all silages received the same pre-treatment, which should help to compensate for possible effects. Once samples entered processing, they were chopped while still frozen, with a Tecator® 1094 homogeniser at 3000 rpm for 30 s. This was decided in order to reduce differences in particle length, as

the original material was, apparently, highly heterogeneous in particle size and plant components (leaves, stems and inflorescences), which could result in subsampling bias and enhanced experimental error.

2.1. Freezing and grinding treatments

Each homogenised sample was subdivided in four subsamples of ca. 320 g and subjected to four treatments: two freezing and two grinding methods. Freezing was performed either by applying liquid nitrogen (LN) or dry ice (DI). LN was applied by direct pouring (700–800 ml) from an insulated container to the silage sample placed in an insulated box. DI was applied with silage placed in a special chamber of synthetic cloth attached to a CO_2 bottle. Freezing to a 'shattering' stage took ca. 1.5 min with LN and 8 min with DI. Frozen silage was ground either with the Tecator homogeniser (Tec) or with a Moulinex® 123 food processor (Mou). Grinding time for Tec and Mou, were 1.2 and 9 min, respectively. DI and Mou required a longer time, since working capacity was insufficient to process the sample amount used in a single batch.

2.2. Spectra collection

Silage from each treatment was split in two halves, each of which was packed in a polyethylene bag (3 μ thick) and sealed. Spectra were taken by reflectance from 400 to 2500 nm, placing the bags in a forage cell which was located in the sample transport module of a NIRSystems 6500 scanning monochromator. Each bag was scanned and then reallocated upside down, so a second scan to the other side could be taken, in order to have four scans or replicates for each silage and treatment. The monochromator was controlled with a PC computer with the software NIRS 3 (ISI, 1992). The instrument was set at 30 readings per scan. Spectra variation was evaluated by the statistic root mean square (RMS, according to software), which is an estimator of the standard error of the difference. The RMS value for each silage was computed from the 30 readings of the four replicates per sample. Once the RMS values were recorded, subsamples spectra were averaged for each sample and saved in a file (*.NIR), one file per treatment.

Principal components (PC) were computed on average spectra, in order to redefine the optical properties of samples, and the Mahalanobis distance of each spectrum with respect to the average spectra was calculated. In this way a structuring of samples according to spectral features is possible and each sample can be graphically placed in a three-dimensional plot defined by any three principal component scores (Shenk and Westerhaus, 1995). The boundaries were defined by the PC of samples of treatment LN-Tec. Although this analysis is intended to evaluate population structuring with hundreds of samples, it was used here as a preliminary way to visualise whether treatments had any segregating effect on the spectra of samples.

2.3. Chemical analysis

Treated and untreated (Control) samples from seven silages were chosen at random and analysed for dry matter by oven (105°C, 24 h) and by reflux distillation with toluene

(DM_{Tol}) according to Dewar and McDonald (1964); crude protein (CP) by micro Kjeldahl and acid detergent fibre (ADF) after Van Soest et al. (1991). CP and ADF determinations were performed on freeze-dried samples ground in a Wiley laboratory mill with a 1-mm screen. CP and ADF were expressed on an oven DM basis.

Effects of freezing and grinding methods on spectral variation (RMS values) and chemical composition (DM, CP and ADF) were evaluated by a two-way (RMS values) or one-way (chemical composition) ANOVA.

3. Results and discussion

3.1. Silage composition

Pasture silages presented a DM_{Tol} content of 266.5 ± 70.0 g kg^{-1} (200.5–387.9 range), a CP content of 126 ± 39.9 g (kg DM)$^{-1}$ (80.5–189.8) and an ADF content of 404.0 ± 21.4 g (kg DM) $^{-1}$ (range 372.0–431.0), thus representing a wide range of compositions.

3.2. Spectral variability

This attribute represented by the statistic RMS did not show any effect ($p>0.05$) of freezing or grinding factors, nor was there any significant interaction among treatments (Table 1).

RMS values were higher than expected, as optimum RMS values in the 100–200 range have been suggested for derivatised spectra, although acceptable values for each product should be set by trial and error (ISI, 1992). The apparently excessive RMS values could have been the result of several factors, including the heterogeneous nature of the samples, differences in particle size which could not be sufficiently controlled by any grinding treatment, the presence of condensation water on the inner surface of the quartz window, traces of effluent accumulated on the inner face of a plastic bag, or the presence for some subsamples of small wrinkles of the plastic film which could not be completely flattened against the quartz, leaving small air pockets which may have affected reflectance of light. Although it is possible to place samples directly in the forage cell without packing them in plastic bags, there is an increase in operation time required for cleaning and drying cells. Besides, when using high moisture, direct cut silages on this type of cell, some silage effluent discharge has been observed, with obvious risk to the instrument.

Table 1
Spectral dissimilarity (RMS values) among sub-samples of silages according to freezing and grinding method

Tretament	Mean	Range	Significance
LN-Tec	415.4 ± 116.1	230–660	NS [a]
LN-Mou	418.3 ± 153.8	209–695	NS [a]
DI-Tec	459.4 ± 104.0	285–642	NS [a]
DI-Mou	402.8 ± 129.4	217–691	NS [a]

[a] $p > 0.05$.

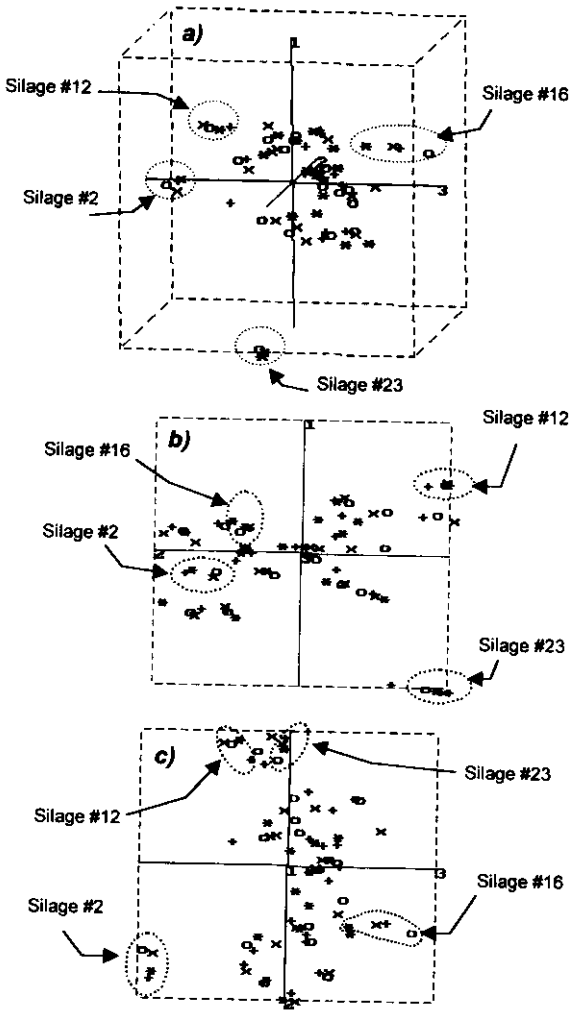

Fig. 1. Symmetry plot of principal component (PC) scores of silage samples subjected to freezing and grinding treatments (see text for details): LN-Tec (×), LN-Mou (+), DI-Tec (○) and DI-Mou (*). In (a) samples are plotted according to the scores of PC 1 (vertical), PC 2 (diagonal) and PC 3 (horizontal); in (b), samples are plotted according to PC 1 (vertical) and PC 2 (horizontal); and in (c), samples are plotted according to PC 2 (vertical) and PC 3 (horizontal). Samples from silages #2, 12, 16 and 23, have been enclosed by a dotted line in order to illustrate spectral similitude among treatments.

With respect to spectral distribution, as affected by treatments, Fig. 1 shows the average spectrum of each sample for each treatment, displayed against the first PC scores of the file correspondent to the treatment LN-Tec.

If processing treatments had important effects on spectral features, samples from the same silage subjected to different freezing or grinding treatments would be plotted apart from each other. This does not seem to be the case, as samples do not reveal segregation

according to treatment. Instead, subsamples from the same silage tend to cluster near one another. The plot of the first three eigenvector scores for each sample produces a symmetry cube (ISI, 1992) in which samples can be displayed from the farthest to the closest position from the mean spectra (Fig. 1(a)). As a three-dimensional display cannot be properly read as such on paper, samples are also displayed according to the first two (Fig. 1(b)), and the second two (Fig. 1(c)), PC scores. As can be seen, samples seem to be mixed instead of segregated according to one particular treatment. To illustrate this point, subsamples from silages #2, 12, 16 and 23, subjected to different treatments, were identified and encircled by a dotted line (Fig. 1(a–c)). It is apparent in this way that samples tend to cluster according to silage and not according to treatment. In this graphical way, a lack of effect of treatments on spectral features is perceptible. This would indicate that samples subjected to different treatments could be pooled together, irrespective of the method of handling, for collection of spectra, although the final test for this hypothesis should be at the validation stage after a calibration is developed for any fraction to be predicted. This stage was not part of this experiment, as a higher number of samples is required for this purpose.

3.3. Chemical composition

No effects on chemical composition were apparent ($p > 0.05$) among treatments (Table 2). A small increase (although not significant) in dry-matter content was apparent with treatments that included the Moulinex food processor (LN-Mou and DI-Mou), probably because grinding time was somewhat longer than with the Tecator homogeniser, since the capacity of the container of the former is smaller. This could lead to some loss of evaporation water in spite of the fact that samples were frozen prior to being processed.

As treatments did not exhibit any significant effect on spectra variability or chemical composition with respect to the fractions analysed here, it may be concluded that any of

Table 2
Toluene dry matter (DM_{Tol}), crude protein (CP) and acid detergent fiber (ADF) content of treated samples

Fraction	Control	LN [b]-Tec [d]	LN [b]-Mou [e]	DI [c]-Tec [d]	DI [c]-Mou [e]	Significance
DM_{Tol} $(g\ kg)^{-1}$						
Mean ± SD	266.5 ± 70.0	265.8 ± 65.0	274.4 ± 56.8	268.3 ± 65.4	271.2 ± 66.5	NS [a]
Range	200.5–387.9	184.0–369.9	200.5–369.3	176.5–368.7	192.5–380.2	
CP $(g\ (kg\ DM)^{-1})$						
Mean ± SD	126.0 ± 39.9	125.0 ± 40.6	126.4 ± 41.3	127.0 ± 41.3	125.4 ± 41.0	NS
Range	80.5–189.8	78.2–190.7	79.6–194.0	79.0–193.2	78.8–192.2	
ADF $(g\ (kg\ DM)^{-1})$						
Mean ± SD	404.0 ± 21.4	407.3 ± 18.6	400.0 ± 24.7	400.6 ± 17.8	404.0 ± 23.0	NS
Range	372.0–431.0	375.9–434.1	360.9–429.5	378.1–429.9	366.9–439.5	

[a] $p > 0.05$.
[b] Liquid nitrogen.
[c] Dry ice.
[d] Tecator homogeniser.
[e] Moulinex food processor.

the methods (treatments) employed could be used for processing silage samples in order to develop calibrations and perform routine NIR analysis, provided that proper, accurate calibrations are produced. It is recommended though, that once a method is chosen, the laboratory should adhere to it in a consistent way from calibration development to routine analysis. Although fresh intact samples without any processing could be used for developing calibrations and subsequent routine analysis, this is especially the case when harvest techniques produce a regular and small particle size. Kennedy et al. (1996) found that good calibrations could be developed for intact silages with chop lengths in the 1.5–2.5 cm range, but with respect to grass up to 20 cm in length, they concluded that prediction errors could be reduced by reducing particle size of these samples. Silages involved in the present study were highly heterogeneous in chop length with particles ranging from 2 to >25 cm, a reflection of the different types of harvester machinery (from flail-type to precision harvesters) employed in the region where silages came from. Under these conditions, a consistent method of sample handling to reduce particle size without affecting composition, prior to recording spectra, can have clear benefits.

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