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Discrimination of different categories of forages harvested from North-western Italy according to near infrared reflectance spectroscopy, chemical composition and *in vitro* digestibility

F. Opsi*,, S. Tassone*, R. Fortina*, S. Andrés*[1], S. López****

*Dipartimento di Scienze Zootecniche, Università degli Studi di Torino, I-10095 Grugliasco-TO, (Italy)
**Instituto de Ganadería de Montaña (CSIC-Universidad de León), 24346 León (Spain)
[1] sonia.andres@eae.csic.es

Abstract. Near infrared reflectance spectroscopy (NIRS), *in vitro* digestibility and fermentation kinetics data were used to discriminate categories of forage quality, based on the method of conservation, forage species, maturity stage or harvest season of 64 forages commonly used in ruminant production systems. Forages used in the study included 40 hay (H) and 24 silage (S) samples of *Lolium multiflorum* L., *Medicago sativa* L., *Zea mays* L. and grassland herbage harvested from 20 dairy cattle farms in Piemonte (N-W Italy). All the samples were scanned in duplicate (400 to 2500 nm) with a NIRSystems 6500 scanning monochromator, and the absorbance data were transformed by a second order derivative before being used in the principal component analysis (PCA). The chlorophyll absorbance in the visible region (650-690 nm) explained most of the variance in the spectra, so maize silage samples were classified apart from grass silage and hay samples. A similar classification was obtained when just the infrared region (1100-2500 nm) was used in the PCA; however, in this latter case the variables contributing to explain most of the response variation along the first two principal components were related to the starch and protein absorbance (2280-2300 nm). Hay samples were highly heterogeneous (different stages of maturity, different harvest seasons or cutting dates within a season, with simple or complex botanical composition), thus precluding discrimination or clustering according to the botanical composition when the absorbance data were used in the PCA. All forage samples could be classified correctly according to the type of conservation (H *vs* S) when combined data of chemical composition, *in vitro* digestibility and gas production kinetics were used in the PCA. However, in agreement with the results obtained for the absorbance data, with the information used in the PCA (gas production, *in vitro* digestibility or chemical data) it was not possible to discriminate different clusters according to the botanical composition, and no categories could be identified within each method of conservation. A proper discrimination of the botanical groups was not possible owing to the high heterogeneity of the samples included in the study, thus causing the overlapping of different clusters.

Keywords. NIRS – Forage – *In vitro* digestibility – Fermentation kinetics.

Différenciation entre diverses catégories de fourrages, récoltés au Nord-Ouest de l'Italie, sur la base de la spectroscopie à reflectance infrarouge (NIRS), la composition chimique et la digestibilité in vitro

Résumé. *La spectroscopie à réflectance infrarouge (NIRS), la digestibilité in vitro et les cinétiques de fermentation ont été utilisées pour analyser la qualité de 64 échantillons de fourrages communément employés dans l'alimentation des ruminants. Les fourrages se distinguaient selon les espèces fourragères, les systèmes de conservation, le stade physiologique et la période de récolte. Les fourrages utilisés dans cette étude ont été : 40 foins (H) et 24 ensilages (S) de* Lolium multiflorum *L.,* Medicago sativa *L.,* Zea mays *L. et d'herbe de prairies récoltés dans 20 exploitations de vaches laitières du Piémont (N-O de l'Italie). Tous les échantillons ont été soumis deux fois au scanner (de 400 à 2500 nm) avec le NIRSystems 6500 scanning monochromator. Les données de l'absorbance ont été transformées par une dérivée de second ordre avant d'être utilisées dans l'analyse des composantes principales (PCA). L'absorbance de la chlorophylle dans la zone visible (650-690 nm) explique une bonne partie de la variance du spectre, c'est pourquoi, les échantillons d'ensilage de mais ont été classés séparément des échantillons d'ensilage d'herbe et des foins. Une classification semblable a été obtenue dans la PCA avec l'emploi de la région infrarouge (1100-2500 nm); cependant, dans ce deuxiè-*

me cas, les variables qui ont contribué à expliquer une bonne partie de la variation le long des deux premières composantes principales ont été l'absorbance de l'amidon et de la protéine (2280-2300 nm). Les échantillons de foins étaient très hétérogènes (différents stades de maturation, différentes périodes de récolte ou de dates de coupe dans la même période de récolte, composition botanique simple ou complexe). Cela a empêché une discrimination ou un ensemble cluster sur la base de la composition botanique avec les données de l'absorbance dans la PCA. En utilisant la PCA, tous les échantillons de fourrages auraient pu être correctement classés selon le système de conservation (H vs *S) avec les données de la composition chimique. Toutefois, en accord avec les résultats obtenus pour les données d'absorbance, aucune des informations utilisées dans la PCA (production de gaz, digestibilité in vitro ou données chimiques) ne portaient à clusters différents sur la base de la composition botanique, et aucune catégorie ne pouvait être distinguée selon la méthode de conservation. La discrimination de groupes botaniques n'a pas été possible à cause de la grande hétérogénéité des échantillons utilisés dans l'étude ce qui a causé la juxtaposition des différents clusters.*

Mots-clés. *NIRS – Fourrage – Digestibilité* in vitro *– Cinétique de la fermentation.*

I – Introduction

Forage quality may have a major effect on animal performance, affecting the voluntary intake and digestibility and, consequently, milk yield and growth of ruminants (Minson, 1990, Getachew *et al.*, 1998) and influencing excretion of undigested nutrients and emission of gases that may have an important impact on the environment (Getachew *et al.*, 2005). Laboratory methods have been developed and refined to provide information on forage quality and to obtain accurate predictions of intake and digestibility from *in vitro* procedures. Recently, a filter bag technique for analyzing *in vitro* dry matter and neutral detergent fibre digestibility was developed by ANKOM Technology Corporation, allowing a large number of samples to be analyzed in a short time (Damiran *et al.*, 2008). Kinetics of rumen degradation of feedstuffs can be studied *in vitro* by means of the gas production technique (Mauricio *et al.*, 1999). These procedures are laborious and require rumen fluid as inoculum. In contrast, NIRS (near infrared reflectance spectroscopy) is a rapid, sensitive and accurate method for the nutritive evaluation of feedstuffs, such as forages (Norris *et al.*, 1976), not requiring the use of animals. This technique is based on the molecular vibrations in the NIR electromagnetic region where the hydrogen is bound to carbon, nitrogen or oxygen, and requires calibration equations to correlate the spectral response to defined reference methods, in order to estimate chemical composition (Norris *et al.*, 1976; García-Ciudad *et al.*, 1993), digestibility (Park *et al.*, 1998) and *in vitro* gas production parameters (Murray, 1993; Andrés *et al.*, 2005). This method can be used as a qualitative tool to discriminate and classify different feedstuffs on the basis of their spectral features (Lister *et al.*, 2000; Prieto *et al.*, 2008).

The aim of this study was to discriminate the nutritive quality of different categories of forages with different botanical composition, method of conservation and maturity stage, by means of near infrared reflectance spectroscopy, chemical composition and *in vitro* digestibility.

II – Materials and methods

The study was carried out with samples of 64 forages commonly used in ruminant production systems. Forages included 40 hay (H group) and 24 silage (S group) samples of *Lolium multiflorum* L., *Medicago sativa* L., *Zea mays* L. and grassland herbage (grass hay 1st, 2nd or 3rd cut, hay single cut or grass silage), collected from 20 dairy cattle farms located in Piemonte region (N-W Italy) during 2008. Samples were oven dried at 60°C for 48 h, then ground in Buhler mill to pass 1 mm screen and analysed for dry matter (DM), ash, crude protein (CP) and ether extract (EE) following the methods of AOAC (1997). Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined with the ANKOM fibre analyser (Ankom Technology Corp., 1997). For the *in vitro* assays six rumen-fistulated Merino sheep were used as donors of ruminal inoculum, fed alfalfa

hay and with free access to water. Ruminal contents were collected before the morning feeding in thermos flasks, taken to laboratory, strained through cheesecloth, kept at 39°C under a CO_2 and diluted (1/4 v/v) with a culture medium containing mineral and buffer solution as described by Van Soest *et al.* (1966). *In vitro* dry matter digestibility (IVDMD) was determined using the ANKOM-DAISY procedure following the approach proposed by Van Soest *et al.* (1966). Samples (0.25 ± 0.01 g) were weighed into F57 Ankom bags with a pore size of 25 µm, heat-sealed and then placed into a jar (5 l volume) containing 2 l buffered rumen fluid. The jars were placed in a DaisyII Incubator (ANKOM Technology Corp., Fairport, NY, USA) at 39°C, with continuous rotation. After 48 h of incubation the jars were emptied and the bags were gently rinsed and dried in an oven at 60°C. Bags were then washed with a neutral detergent solution at 100°C during 1 h and rinsed with distilled water into the fibre analyzer. Considering the amount of NDF incubated, *in vitro* NDF degradation (IVNDFD) could be estimated. Four incubation runs were carried out in different weeks giving four single observations per sample. *In vitro* gas production measurements were conducted using a pressure transducer as described by Theodorou *et al.* (1994), in which 0.50 ± 0.01 g of sample was incubated in a 120 ml serum bottle containing 50 ml of diluted rumen fluid. Bottles were sealed, shaken and placed in the incubator at 39°C. The head-space gas pressure released upon fermentation of feed was measured using a transducer, at incubation times of 3, 6, 9, 12, 16, 21, 26, 31, 36, 48, 60, 72, 96, 120 and 144 h after inoculation time. Gas volume was estimated from pressure measurements using the equation suggested by Lopéz *et al.* (2007). Three incubation runs were performed in different weeks, using in each of them two bottles per sample. Blanks were used to compensate for gas production in the absence of substrate. ANKOM-DAISY procedure with 144 h of continuous incubation was used to estimate the potential DM disappearance (D144). The exponential model proposed by France *et al.* (2000) was fitted to gas production data: $G = A\ [1 - e^{-c(t-L)}]$, where G (ml/g DM) is the cumulative gas production at time t, A (ml/g DM) the asymptotic gas production, c (h^{-1}) the fractional rate of fermentation and L (h) is the lag time. The extent of degradation in the rumen (ED), for a rate of passage (k) of 0.033 h^{-1} (characteristic of sheep fed a forage diet of maintenance level), was estimated using the equation suggested by France *et al.* (2000): $ED = (D144 \times c \times e^{-kL}) / (c + k)$.

All samples were subsequently scanned in duplicate using a scanning monochromator NIRSystems (FOSS, Silver Spring, MD, USA) and spectra were collected in the visible region (vis) and NIR region (400-2500 nm) at 2 nm intervals. Absorbance was calculated as log (1/R), where R is measured reflectance. The mean spectrum was used for each sample, and different mathematical treatments of the spectra, based on first or second order derivatives, were applied. Raw spectra were noticeably improved when using the second derivative transformation, so that finally these data were used in the principal component analysis (PCA).

III – Results and discussion

Ranges of values on chemical composition of forages are presented in Table 1. The dry matter content was different for both methods of conservation, with values ranging between 819 and 929 g/kg in hays and from 210 to 639 g/kg in silages, although some hays and silages were prepared from the same source material. The CP, NDF and ADF contents differentiated maize silage from grass silages and hay samples, with narrower ranges of 68-88 g CP/kg DM, 361-528 g NDF/kg DM and 207-343 g ADF/kg DM when compared to the other forages. Alfalfa forage, either as hay or silage form, showed a higher lignin content than other forages, with values ranging from 53 to118 g/kg DM.

Ranges of *in vitro* digestibility coefficients and parameters of fermentation are presented in Table 2. All variables observed did not discriminate the forages according to the botanical composition and type of conservation, because all the forages showed similar ranges of values, regardless their botanical composition or method of conservation.

Data of chemical composition, *in vitro* digestibility and gas production kinetics (a), and those derived from NIR spectra of samples (1/R), improved by second-order derivative to obtain a bet-

ter resolution of raw spectra (b), were used in the principal component analysis (PCA) to obtain the corresponding scores of samples for the first two principal component (PC1-PC2) in a coordinate axis system, as is shown in Fig. 1.

Table 1. Ranges in chemical composition (g/kg DM) of forages

Forages	n	Dry matter	Ash	Crude protein	Ether extract	NDF	ADF	ADL
Alfalfa hay	8	870-929	62-93	81-170	10-21	542-694	409-526	53-118
Italian ryegrass hay	6	819-915	67-88	64-89	13-22	583-690	344-413	33-58
Grass hay 1st cut	14	822-913	64-104	58-132	10-31	542-668	325-522	26-103
Grass hay 2nd cut	6	851-887	56-91	73-158	15-36	544-676	294-404	25-48
Grass hay 3rd cut	3	848-869	69-101	132-157	25-31	591-624	333-360	34-47
Grass hay single cut	2	876-909	60-81	44-127	10-29	633-638	383-399	45-47
Italian ryegrass silage	4	285-639	98-149	96-166	16-29	512-660	279-415	24-55
Grass silage	3	210-539	107-158	113-129	21-36	567-614	315-401	37-47
Maize silage	17	252-375	39-48	68-88	28-45	361-528	207-343	17-38
Alfalfa silage	1	428	141	145	22	550	363	84

NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin.

Table 2. *In vitro* digestibility (g/g dry matter) and parameters of fermentation (ml/g dry matter)

Forages	n	*In vitro* digestibility (ranges)				Parameters of fermentation (ranges)		
		IVDMD	IVNDFD	D144	ED	G24	*A*	*c* (h^{-1})
Alfalfa hay	8	0.595-0.688	0.361-0.532	0.618-0.740	0.363-0.453	162-199	239-292	0.034-0.066
Italian ryegrass hay	6	0.641-0.855	0.480-0.757	0.683-0.871	0.336-0.440	153-205	284-322	0.032-0.049
Grass hay								
1st cut	14	0.566-0.796	0.341-0.654	0.619-0.848	0.306-0.474	90-212	211-331	0.024-0.049
2nd cut	6	0.668-0.775	0.401-0.621	0.711-0.835	0.386-0.480	169-220	283-331	0.037-0.047
3rd cut	3	0.533-0.772	0.252-0.615	0.587-0.791	0.267-0.460	118-200	243-300	0.028-0.046
single cut	2	0.698-0.720	0.523-0.561	0.729-0.747	0.382-0.396	168-209	297-343	0.040-0.036
Italian ryegrass silage	4	0.642-0.831	0.300-0.697	0.710-0.857	0.361-0.488	108-211	191-327	0.035-0.049
Grass silage	3	0.712-0.734	0.530-0.538	0.780-0.822	0.431-0.463	172-198	275-305	0.041-0.045
Maize silage	17	0.616-0.761	0.128-0.443	0.715-0.812	0.375-0.455	188-252	310-372	0.043-0.059
Alfalfa silage	1	0.677	0.412	0.728	0.434	169	245	0.049

IVDMD, in vitro dry matter digestibility; IVNDFD, in vitro neutral detergent fibre digestibility; D144, in vitro dry matter disappearance after 144 h of incubation; ED, extent of degradation; G24, cumulative gas production at 24 h; A, asymptotic gas production; c, fractional rate of fermentation.

When combined data of chemical composition, *in vitro* digestibility and gas production kinetics were used in PCA analysis two different clusters could be observed (Fig.1a), one for hays (identified as H group) and another one grouping silage samples (S group). This discrimination could be attributed to differences between both types of forages in chemical composition according to data presented in Table 1. The botanical composition did not allow the discrimination of forages. The grouping based on absorbance data with second-order derivative transformation is presented in Fig. 1b. Regarding these data, just the maize silage samples (A group) could be discriminated from the rest of samples (B group), with no clear separation in the last group related to forage conser-

vation (hay vs. silage) or botanical composition. Analysing the spectra profiles, it seemed that the variables explaining a greater proportion of the variance were related to the colour, with chlorophyll absorbance at 650-690 nm (in the visible region). A similar classification was obtained when just the infrared region (1100-2500 nm) was used in the PCA; however, in this latter case the variables contributing to explain most of the response variation along the first two principal components were related to the starch and protein absorbance (2280-2300 nm).

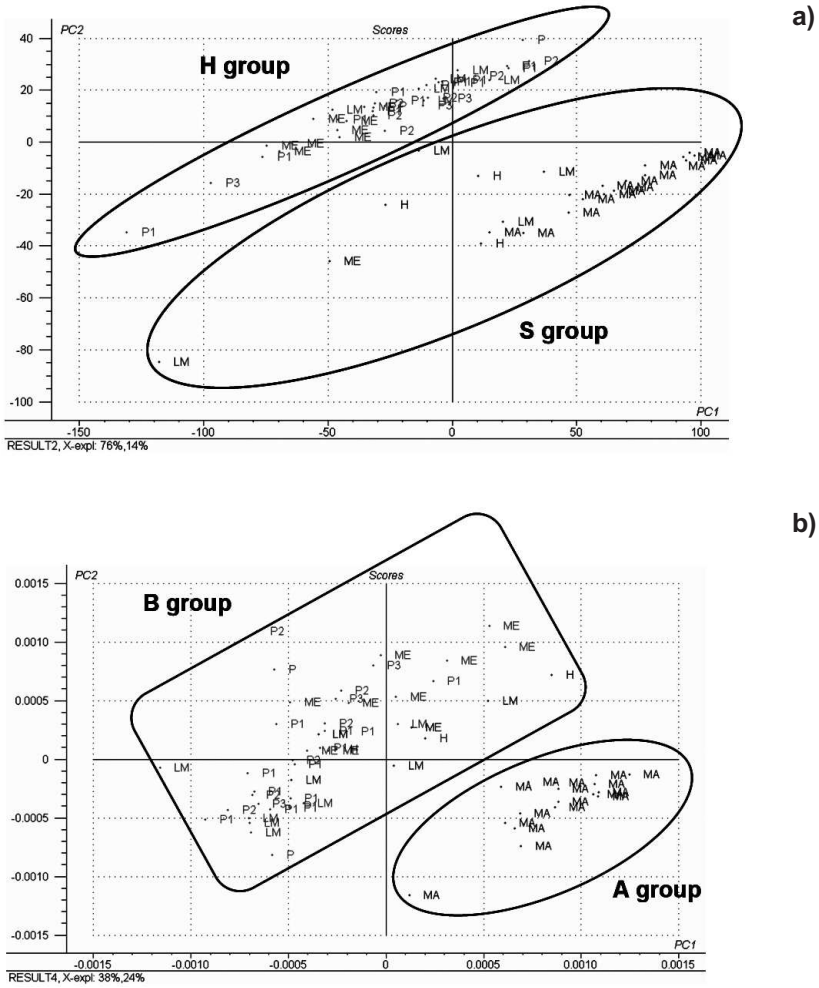

Fig. 1. Plots of samples in the principal components (PC1 – PC2) calculated using either (a) data of chemical composition, *in vitro* digestibility and parameters of fermentation kinetics, or (b) second-order derivative data from average NIR spectra. Forages were alfalfa (ME), Italian ryegrass (LM), P1, grass hay from 1st (P1), 2nd (P2), 3rd (P3) or a single (P) cut, grass silage (H) and, maize silage (MA).

IV – Conclusions

Forage samples could be classified correctly according to the type of conservation (H *vs* S) when combined data of chemical composition, *in vitro* digestibility and gas production kinetics were used in the PCA. However, using absorbance data in the PCA analysis it was not possible to discriminate different clusters according to the botanical composition, and no categories could be identified within each method of conservation. A proper discrimination of the botanical groups was not possible owing to the high heterogeneity of the samples included in the study, thus causing the overlapping of different clusters.

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