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in

Baumont R. (ed.), Carrère P. (ed.), Jouven M. (ed.), Lombardi G. (ed.), López-Francos A. (ed.), Martin B. (ed.), Peeters A. (ed.), Porqueddu C. (ed.).
Forage resources and ecosystem services provided by Mountain and Mediterranean grasslands and rangelands

Zaragoza : CIHEAM / INRA / FAO / VetAgro Sup Clermont-Ferrand / Montpellier SupAgro
Options Méditerranéennes : Série A. Séminaires Méditerranéens; n. 109

2014
pages 375-378

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Abstract. Near infrared reflectance spectroscopy (NIRS) was evaluated as an alternative method to gas chromatography for the quantitative analysis of fatty acids (FA) in samples of six annual legume species grown as winter crops in Galicia (NW Spain) and harvested at different dates in spring. Spectral data from both whole plant and morphological fractions (leaves, petioles, stems and inflorescences) samples of crimson clover (*Trifolium incarnatum* L.), balansa clover (*T. michelianum* Savi.), persian clover (*T. resupinatum* L.), ssp. *resupinatum* and *majus*, arrowleaf clover (*T. vesiculosum* Savi.) and French serradella (*Ornithopus sativus* Brot.) were collected in the wavelength range of 1100-2500 nm. Chromatographic data was regressed on spectral data to develop calibration equations for FA composition, sum of total FA (TFA), saturated FA (SFA), unsaturated FA (UFA), and polyunsaturated FA (PUFA). Equations showed good or excellent coefficients of determination (R^2) for calibration (0.80-0.93), except for C17:0. It can be concluded that NIRS analysis is a useful tool for the prediction of fatty acid composition of the annual legumes studied.

Keywords. Forage legumes – Fatty acids – NIRS.

Prédiction de la composition en acides gras de fourrages annuels de trèfles et serradelle par spectroscopie dans l'infrarouge proche

Résumé. *La spectroscopie dans le infra rouge proche (NIRS) a été évaluée comme une méthode alternative à la chromatographie en phase gazeuse pour l'analyse quantitative des acides gras au sein de six échantillons de légumineuses annuelles cultivées comme culture d'hiver en Galice (NO Espagne) et récoltées à différentes dates au printemps. Les données spectrales obtenues à partir soit de la plante dans son ensemble, soit de fractions morphologiques (feuilles, pétioles, tiges et inflorescence) de trèfle incarnat, trèfle Balansa, trèfle de Perse, trèfle vésiculeux et serradelle française, ont été acquises dans un intervalle de longueurs d'onde compris entre 1100 et 2500 nm. Les données chromatographiques ont été corrélées avec les données spectrales dans le but de développer des équations de calibration pour déterminer la composition en acides gras, la somme des acides gras totaux (AGT), saturés (AGS), insaturés (AGI) et polyinsaturés (AGPI). Les équations de calibration ont montré de bons voire d'excellents coefficients (R^2) de corrélation, sauf dans le cas de l'acide C17:0. L'analyse NIRS constitue une méthode très utile pour la prédiction de la composition en acides gras des légumineuses fourragères annuelles étudiées.*

Mots-clés. *Légumineuses fourragères – Acides gras – NIRS.*

I – Introduction

The dairy cow farms of Galicia (NW Spain) produce 2.2 million of tonnes of milk, accounting for about 40% of total dairy production and more than half of dairy cow producers in Spain (MARM, 2010). In recent years, the number of farmers interested about production of differentiate milk have been increased, with feeding system based on forage legumes, due to their potential for enhance the proportion of polyunsaturated fatty acids (PUFA) in milk fat (Dewhurst *et al*., 2003).

Therefore for evaluation of profile fatty acids (FA) of some annual forage legumes species as winter crops for silage in Galicia, arising the need of having reliable methods for the routine evaluation of fatty acids composition for both research and advisory purposes.

Near infrared reflectance spectroscopy (NIRS) is an alternative to standard analytical methods for determining nutritive value of forages and has become widely recognized as a valuable tool in the accurate determination of the chemical composition of a wide range of forages (Shenk and Westerhaus, 1985). The key to successful use of the NIRS technique is to develop a calibration model, based in a large calibration database which adequately represents the characteristics of the forage problem samples to be predicted.

In the present study, it is evaluated the potential use of NIRS as a alternative method to gas chromatography for the quantitative analysis FA in samples of six annual legume species grown as winter crops in Galicia (Valladares *et al*., 2012) and harvested in different dates in spring for silage as the first step in providing a robust, fast and inexpensive laboratory method for estimating FA composition of these species.

II – Materials and methods

This work was carried out with samples of six annual legume species harvested at six different dates during the first and second spring growth of the year 2011 (15 March-24 May and 26 April-5 July, respectively) with 10 replications, in an experiment carried out at the Centro de Investigacións Agrarias de Mabegondo (A Coruña, Galicia, Spain). Moreover, the fresh whole plants of all legumes species, were harvested at five harvest dates (3 April-29 May) during the first spring growth of the same experiment in year 2012 (1 replicate), the legumes were separated by hand into morphological fractions: leaves, petioles, stems and inflorescences. The species studied were Crimson clover (*Trifolium incarnatum* L. cv. Viterbo), Balansa clover (*T. michelianum* Savi. cv. Bolta), Persian clover (*T. resupinatum* L. ssp. *resupinatum* cv. Kyambro and ssp. *majus* cv. Maral), Arrowleaf clover (*T. vesiculosum* Savi. cv. Zulu) and French serradella (*Ornithopus sativus* Brot. cv. Margurita), this specie was not harvested for separation into morphological fractions. Dry matter (DM) content of fresh samples was determined by oven-drying at 80ºC for 16 hours, ground in a Christy-Norris hammer mill to 1 mm and sample spectra were recorded in a Foss NIR Systems 6500 monochromator (spectofotometric NIRSystems 6500 (FOSS NIRSystems, Inc., Silver Spring, Washington, USA). Two aliquots of each sample were scanned in a spinning circular cup with a quartz window of 37.5 mm diameter, at 2 nm intervals in the wavelength range of 1100-2500 nm. The spectrum of each sample was the average of the two sub-samples. Initially, all spectral data were recorded as the log 1/reflectance (log 1/R values). Samples with extreme (i.e. outliers) or very similar spectra were excluded from the calibration data set, unless they were thought to provide relevant information (Shenk and Westerhaus, 1996). Data were processed using the software Win ISI Version 1.5 (Infrasoft International, Port Matilda, USA, 2000).

From total samples (n = 820) the most representative spectra were selected using the SELECT algorithm included in the WinISI software. Fatty acids of forage selected samples (n=190, 107 whole plant and 83 fractions) were extracted using a modified version of the direct transesterification method developed by Sukhija and Palmquist (1988) performing a simultaneous extraction and methylation with methanolic hydrochloric acid. The FA methyl esters were detected and quantified by gas chromatography coupled with a flame ionization detector. All parameters were reported on a DM basis and analysed in duplicate.

Calibration equations were obtained using a Modified Partial Least Squares Regression (MPLS) regression technique (Martens and Naes, 1987). This regression technique requires cross-validation to prevent over-fitting, obtaining validation errors by partitioning the calibration set into several groups and pooling them into a standard error of cross-validation. MPLS of reference val-

ues on the second derivative of standard normal variate and de-trended spectra (Barnes *et al*. 1989) was regression method for all the determinations. The statistics parameters used to test the performance of the calibration equations were the standard errors (SEC and SECV) and coefficients of determination (R^2 and r^2) obtained in the calibration and cross validation steps, respectively. The Range to Error ratio (RER) of cross validation, as defined by the range of the population's reference values divided by the corresponding SECV was also considered since it is a useful statistic to test the accuracy of the calibration models (Williams and Sobering, 1996).

III – Results and discussion

The characteristics of the reference values of the calibration data set and the statistics to describe the quality of NIRS calibration equations developed are shown in Table 1. The variability in their composition was found to be adequate for developing initial NIRS calibrations.

Table 1. NIRS statistics of the calibration equation used for the prediction of fatty acid (FA) composition, sum of total FA (TFA), saturated FA (SFA), unsaturated FA (UFA), and polyunsaturated FA (PUFA) of whole plant and morphological fractions of the annual forage legume species

Constituent	N	Mean	SD	Range	SEC	R^2	SECV	r^2	RER
C12:0	177	0.086	0.078	0.000-0.310	0.027	0.88	0.035	0.82	8.77
C14:0	175	0.028	0.025	0.000-0.098	0.012	0.75	0.016	0.65	6.22
C15:0	183	0.027	0.026	0.000-0.097	0.009	0.87	0.011	0.82	8.55
C16:0	183	1.822	0.783	0.117-4.193	0.009	0.87	0.419	0.82	9.72
C16:1	183	0.056	0.015	0.000-0.065	0.295	0.86	0.039	0.73	1.65
C17:0	158	0.026	0.019	0.000-0.082	0.009	0.64	0.010	0.16	8.15
C18:0	182	0.181	0.124	0.000-0.540	0.008	0.85	0.066	0.74	8.20
C18:1n9c	182	0.123	0.079	0.000-0.332	0.051	0.83	0.072	0.74	4.63
C18:2n6c	185	1.817	0.810	0.000-4.214	0.367	0.80	0.482	0.65	8.75
C20:0	163	0.139	0.068	0.000-0.295	0.023	0.88	0.052	0.66	5.63
C18:3n6	183	0.056	0.050	0.000-0.179	0.015	0.91	0.026	0.74	7.02
C20:1	181	0.024	0.017	0.000-0.074	0.008	0.80	0.010	0.70	7.62
C18:3n3	183	5.899	4.032	0.092-17.974	1.101	0.93	1.310	0.90	13.65
C22:0	181	0.106	0.073	0.000-0.323	0.031	0.82	0.039	0.73	8.23
C22:1n9	132	0.004	0.004	0.000-0.014	0.001	0.91	0.002	0.71	5.83
C20:3n3	190	0.038	0.028	0.000-0.121	0.013	0.78	0.016	0.68	7.44
C20:4n6	179	0.039	0.029	0.000-0.122	0.014	0.80	0.016	0.71	7.50
C24:0	180	0.150	0.098	0.000-0.435	0.035	0.88	0.050	0.75	8.75
TFA	188	10.677	5.504	1.873-25.784	1.854	0.89	2.170	0.84	11.02
SFA	182	2.623	1.214	0.264-6.218	0.409	0.89	0.581	0.77	10.24
UFA	189	8.055	4.504	1.153-20.825	1.431	0.90	1.717	0.85	11.46
PUFA	184	7.848	4.369	1.051-20.572	1.397	0.90	1.661	0.86	11.75

SD = standard deviation. SEC = standard error of calibration. SECV = standard error of cross validation. R^2 and r^2 = coefficient of determination in calibration and cross validation. RER = Range/SECV.

The values obtained for the coefficients of determination (R^2) of calibration ranged from 0.64 to 0.93, according to the criteria proposed by Shenk and Westerhaus (1996), an R^2 value greater than 0.90 indicates an excellent quantitative precision, while a value between 0.70 and 0.90 is described as a good quantitative precision, therefore, the equations developed in this work showed good or excellent coefficients of determination for calibration, except for C17:0. On the

other hand, according to the guidelines utilized by Williams and Sobering (1996) calibrations with RER>10, are acceptable for quantitative prediction, the RER values were greater than 10 for the more prevalent fatty acids (C18:3n3, TFA, SFA, UFA and PUFA). Ours results showed values for coefficients of determination of calibration and cross validation lower than those reported by Foster *et al.* (2006).

IV – Conclusions

This work demonstrates the possibilities to use NIRS for the prediction of fatty acid composition of the six annual legumes studied. It is nevertheless advisable to check the equation in blind tests on open sets into the future and to include new samples to increase the robustness of the predictions and expand it to other legume species.

Acknowledgments

The authors wish to acknowledge the financial support received from the INCITE of Xunta de Galicia (Project PGIDIT09MRU012E) and the dairy company Leyma Central Lechera. S.A.

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