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Vibrational spectroscopy to predict *in vitro* digestibility and the maturity index of different forage crops during the growing cycle and after freeze- or oven-drying treatment

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ABSTRACT

The aims of the study were to utilize vibrational spectroscopy as a rapid predictive tool of forage quality; to compare two preparation methods, freeze- (FD) *vs.* oven-dried (OD); to focus on the progression of intra- and inter-family maturity by adopting a multivariate crop maturity index (CMI) based on composition, digestibility and tillage traits. A panel of forages ($n = 158$) composed of 12 crops (borage, chia, false flax, flax, galega, hemp, perilla, quinoa, ravizzone, safflower, sunflower and white lupin) derived from 8 botanic families, sampled at different vegetative stages, and which were FD or OD, were examined. Two spectrometers were used at different spectral regions: a Perkin Elmer IdentiCheck™ (PE, B-band, 714–1025 nm; C-band, 1026–2500 nm, D-band, 2501–3333 nm) and a portable Analytical Spectral Device (ASD, A-band, 350–713 nm, UV–Vis; B-band, as above). The absorption spectra were constantly higher in the OD samples and showed very high discriminability. The average prediction response (RPD, defined as the performance-deviation ratio) was better with the PE instrument, because of its enhanced band capabilities. However, the response over the spectral regions differed on the basis of which instrument was used and according to the preparations. The ASD instrument was more efficient in the B-band, for the OD preparation and better than PE in the pooled calibration (RPD:1.63 *vs.* 1.20; P=0.0005). A significant superiority in the NIR C-band for the FD preparation was observed (RPD: 2.46 *vs.* 1.95; P=0.004), while, unexpectedly, the MIR D-band was 25% more performing (RPD: 2.78 *vs.* 2.21; P=0.0005). The ash, the neutral detergent fiber (NDFom) and its indigestible part (INDF) were placed at the highest prediction rank in both instruments, albeit at different precision levels, caused by the different instrumental capabilities, with an overall

Abbreviations: ADFom, acid detergent fiber expressed exclusive of residual ash; ASD, Analytical Spectral Device; CMI, crop maturity index; CP, crude protein; *D*, days after seeding; DM, dry matter; DNDF, digestible neutral detergent fiber; EE, ether extract; FD, freeze-dried; GE, gross energy; INDF, indigestible neutral detergent fiber; IVTD, *in vitro* true digestibility; MIR, medium infra-red; MSE, mean square error; NDFD, *in vitro* neutral detergent fiber digestibility; NDFom, neutral detergent fiber expressed exclusive of residual ash; NIRS, near infrared spectroscopy; OD, oven-dried; OM, organic matter; PE, Perkin Elmer; RPD, ratio-performance deviation; r^2v, *r*-square of reciprocal validation; SD, standard deviation; SECV, standard error in cross-validation; VC, variation coefficient; 1-VR, *r*-square of internal cross-validation.

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1-VR avg. of 0.81 (1-VR, defined as the *r*-square of internal cross-validation). In a composite FD-OD equation, the best prediction was made by the INDF (1-VR of 0.91 and 0.88 for the PE and ASD instruments, respectively). The worst performances were observed for the digestible neutral detergent fiber (DNDF) prediction. The CMI was influenced by the INDF (R^2 0.91) and was accurately predicted by vibrational spectroscopy (RPD 5.2 and 2.9 for PE and ASD, respectively). CMI was able to summarize the botanical differences and highlight a rank between the eight families from the less mature pole: *Boraginaceae* and *Chenopodiaceae* < *Lamiaceae* < *Asteraceae* and *Fabaceae* < *Cannabaceae* < *Brassicaceae* < *Linaceae* (the most mature type). Four particular wavelengths have been identified as they are related to the CMI, namely: 701 (red), 905, 2451 and 2799 nm, respectively, in the A, B, C and D bands.

1. Introduction

Digestibility is the most common nutritive parameter used in feeding standards for ruminants (Coleman and Moore, 2003; NRC, 2001), and is the basal unit when evaluating the nutritive value of forage (Jancik et al., 2011; Wang et al., 2009). In fact, the accurate estimation of forage digestibility is a prerequisite for diet formulation, the economic evaluation of forages and the prediction of animal responses (Ricci et al., 2009). Digestibility can be estimated through several techniques, whose results can differ considerably (Huhtanen et al., 2006). Forage digestibility can be studied *in vivo*, *in situ* and *in vitro* (Cone et al., 1999). Chemical composition parameters have also been used to estimate the digestibility of forages, since it is well known that the structure and thus the components of the plant, vary as the stage of maturity advances. Given that *in vivo* determinations of digestibility are laborious, expensive and difficult to standardize, *in situ* and *in vitro* techniques have been developed (Gosselink et al., 2004; Stern et al., 1997). Much of this work has been done on ruminant species, and has provided estimates that are closely correlated to *in vivo* digestibility values (Earing et al., 2010; Goldman et al., 1987; Stern et al., 1997). Over the years, various procedures have been developed and modified to determine digestibility. Recently, an efficient alternative to the traditional *in vitro* method (Tilley and Terry, 1963) has been developed by Ankom Technology (Fairport, NY, USA). This *in vitro* filter bag technique, which uses Daisy^{II}, is a reliable and simple technique that is easier and less time-consuming than the conventional *in vitro* technique (Damiran et al., 2008; Holden, 1999; Mabjeesh et al., 2000; Truijllo et al., 2010). It has been shown to increase labor efficiency and precision. However, the technique involves the *inoculum* of rumen fluid, which is the main factor that can introduce errors into neutral detergent fiber digestibility assays (Goeser and Combs, 2009).

A valid alternative method is the physical, rapid and non-destructive vibrational spectroscopy technique, which represents a radical departure from conventional chemical methods, in that the whole matrix derived from crops is characterized in terms of its absorption properties. In fact, all organic molecules constantly vibrate, and continue to absorb energy from incoming photons to increase their vibrations. Vibrational spectroscopy, based on the medium infra-red regions (MIR, 2500–25,000 nm) deals directly with fundamental vibrations and specific absorptions; the overtones that rebound in the near infra-red (NIR), the visible (Vis) and in the UV regions (2500–350 nm) are instead gained from powerful chemometrics to deconvolute intrinsic chemical information. The technique offers the advantages of simplicity, speed, no chemical waste and more cost-effective prediction, even though it requires laborious calibration procedures and the choice of the data treatment is complex. NIRS has revolutionized the nutritional characterization of animal feeds (Coleman and Moore, 2003; Givens and Deaville, 1999), and has been shown to be able to predict *in vivo* digestibility (Deaville et al., 2009; Decruyenaere et al., 2009; Landau et al., 2006). The neighboring spectral regions, such as UV, Vis, or MIR, are instead rarely used in animal nutrition studies.

The main objective of this study was to evaluate the potential of vibrational spectroscopy applied to the available electromagnetic radiation (UV–MIR) to predict the chemical composition, and *in vitro* digestibility assessed by the Daisy^{II} system, adopting two different instruments and two sample preparation methods (oven-dried, or freeze-dried). Because of the availability of a wide range of botanically variable grass species, the second aim of the study was to focus on inter-species and family variations. Thus, a linear multivariate CMI, which is able to synthesize information from all the available parameters, has been formulated for the forages; the CMI could also be predicted by means of vibrational spectroscopy.

2. Materials and methods

2.1. Plant material and chemical analyses

Twelve sets of field data of borage (*Borago officinalis* L.), galega (*Galega officinalis* L.), false flax (*Camelina sativa* L.), flax (*Linum usitatissimum* L.), hemp (*Cannabis sativa* L.), chia (*Salvia hispanica* L.), safflower (*Carthamus tinctorius* L.), sunflower (*Helianthus annuus* L.), white lupin (*Lupinus albus* L.), perilla (*Perilla frutescens* L.), ravizzone (*Brassica campestris* L. *var. Oleifera*) and quinoa (*Chenopodium quinoa* Willd.), collected in various studies from 2002 to 2010, have been used in this experiment. Overall, 158 samples of these green crops were collected at progressive morphological stages, up to sub-maturity, in order

Table 1
Species, botanic family, number of samples and of stages, harvest range in days after seeding at the first stage ($D_{\text{first stage}}$), the last stage ($D_{\text{last stage}}$) and the final morphological stage.

Name	Species	Botanic family	Samples	Stages	$D_{\text{first stage}}$	$D_{\text{last stage}}$	Final stage
Borage	*Borago officinalis*	*Boraginaceae*	9	4	35	75	Early seed
False flax	*Camelina sativa*	*Brassicaceae*	15	5	42	70	Ripe seed-pod
Chia	*Salvia hispanica*	*Lamiaceae*	14	5	42	111	Budding
Hemp	*Cannabis sativa*	*Cannabaceae*	12	4	45	65	Early flowering
Safflower	*Carthamus tinctorius*	*Asteraceae*	15	5	36	62	Early flowering
Galega	*Galega officinalis*	*Fabaceae*	12	4	48	70	Budding
Sunflower	*Helianthus annuus*	*Asteraceae*	15	5	36	62	Late flowering
Flax	*Linum usitatissimum*	*Linaceae*	15	5	45	99	Seeding
White lupin	*Lupinus albus*	*Fabaceae*	8	4	50	85	Young green pod
Perilla	*Perilla frutescens*	*Lamiaceae*	14	5	42	104	Early flowering
Quinoa	*Chenopodium quinoa*	*Chenopodiaceae*	17	6	49	141	Grain fill
Ravizzone	*Brassica campestris*	*Brassicaceae*	12	4	49	70	Ripening

to provide a large variability of quality parameters (Table 1). The samples were prepared and analyzed for their chemical constituents, as reported in Peiretti et al. (2013). Part of the fresh crop was chopped, frozen, freeze-dried, and ground in a Cyclotec mill (Tecator, Herndon, VA, USA) to pass a 1 mm screen. A second aliquot was oven dried at 90 °C for 24 h for dry matter (DM) determination, while a third major aliquot of the plant samples was dried in a forced-draft oven at 60 °C up to constant weight, air-equilibrated, ground in a Cyclotec mill and stored for later analysis. The dried samples were analyzed to determine the total N content according to the Dumas method, using a macro-N Nitrogen analyzer (Foss Heraeus Analysensysteme, Hanau, Germany), ash by ignition at 550 °C, neutral detergent fiber (NDFom) without sodium sulfite and α-amylase, and acid detergent fiber (ADFom) as described by Van Soest et al. (1991). Gross energy (GE) was determined using an adiabatic calorimeter bomb (IKA C7000, Staufen, Germany).

2.2. *In vitro* digestibility and connected variables

The freeze-dried samples were analyzed to determine the *in vitro* true digestibility (IVTD) and *in vitro* neutral detergent fiber digestibility (NDFD) using the Daisy^{II} Incubator (Ankom Technology Corp., Fairport, NY, USA), according to Robinson et al. (1999). The *in vitro* rumen incubations were performed in two consecutive fermentative runs. Ground samples (250 mg) were inserted into filter bags (Ankom F57 bags), which were then sealed. Digestion jars were filled with pre-warmed (39 °C) buffer solutions (266 mL of solution A: KH_2PO_4 10 g/L, $MgSO_4{\cdot}7H_2O$ 0.5 g/L, NaCl 0.5 g/L, $CaCl_2{\cdot}2H_2O$ 0.1 g/L, and urea 0.5 g/L; 1330 mL of solution B: Na_2CO_3 15.0 g/L, $Na_2S{\cdot}9H_2O$ 1.0 g/L) and placed into the incubator. Rumen liquor was collected in a slaughterhouse from rumen contents obtained from cattle (two runs) from the same farm fed a fiber-rich diet (Peiretti et al., 2013). Four hundred milliliter of liquor (filtered through two layers of cheesecloth) was introduced into each jar, together with the filter bags. After 48 h of incubation, the bags were removed, rinsed thoroughly with cold tap water and immediately analyzed for the NDFom content with the Ankom^{200} Fiber Analyzer, following the Ankom Technology Method. The replicated analyses were averaged by sample.

IVTD (g/kg DM) was calculated using the following equation:

$$1000 - (W_3 - (W_1 \cdot C_1)) \times 1000/(W_2 \cdot \text{DM}),$$

where W_1 is the filter bag weight, W_2 is the sample weight, W_3 is the final weight (filter bag + residue) after *in vitro* and sequential treatment with a neutral detergent solution, C_1 is a comparison of the blank filter bag weight before and after the digestion treatment and DM is the dry matter content of the samples.

NDFD (g/kg NDFom) was calculated using the following equation:

$$1000 - (W_3 - (W_1 \cdot C_1)) \times 1000/(W_2 \cdot \text{NDFom})$$

where W_1 is the filter bag weight, W_2 is the sample weight, W_3 is the final weight (filter bag + residue) after *in vitro* and sequential treatment with a neutral detergent solution, C_1 is a comparison of the blank filter bag weight before and after the digestion treatment, and NDFom is the neutral detergent fiber content of the sample.

The total neutral detergent fiber is derived from the sum of the digestible neutral detergent fiber (DNDF, g/kg DM) and the indigestible neutral detergent fiber (INDF, g/kg DM), according to the neutral detergent fiber digestibility coefficient. The sum and the two *addendi* are statistically linked, and the neutral detergent fiber variance is:

$$\text{Var(NDFom)} = \text{Var(DNDF)} + \text{Var(INDF)} + 2\text{Cov(DNDF, INDF)}$$

Table 2
Chemical characteristics and digestibility features of the twelve crops and phenological effects of the days after seeding (*D*).

Species	DM	Ash	CP	GE	ADFom	NDFom	IVTD	NDFD	DNDF	INDF
Borage	89e	220a	145ab	14.8g	281e	329e	895a	691bc	224d	104f
False flax	204a	88f	132b	17.4b	393b	465c	726cd	435e	192e	273ab
Chia	133cd	128cd	122b	16.3e	312de	407d	877ab	707ab	285bc	122f
Hemp	207a	116de	152ab	16.8d	345c	483c	805b	598c	288b	194c
Safflower	139cd	126cd	162a	16.9cd	320cd	425d	815b	597c	240d	184cd
Galega	150bc	105e	165a	17.5b	289de	421d	842b	638c	264cd	157de
Sunflower	106de	142bc	104c	15.8f	388b	423d	832b	632c	259cd	164de
Flax	215a	68g	138b	18.6a	460a	564a	704d	490de	268bc	295a
White lupin	101e	133bcd	164a	17.0cd	327cd	393d	848b	609c	241d	151de
Perilla	120d	134bcd	143ab	16.3e	315de	441cd	890a	759a	332a	109f
Quinoa	132d	198b	135b	15.0g	284e	464c	858ab	701b	322a	141ef
Ravizzone	165b	91f	101c	17.2c	391b	515b	749c	517d	264cd	250b
R^2 (full-model 1)	0.78	0.88	0.49	0.91	0.75	0.78	0.78	0.73	0.65	0.78
VC %	17	13	23	2	12	9	6	13	11	25
RMSE	25.4	16.5	31.4	0.35	40.4	40.4	4.60	8.01	30.3	45.7
Mean	150	128	138	167	345	450	81.7	61.4	268	182
P (full-model 1)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
B (regression)	1.343	−0.854	−1.112	0.013	2.425	2.300	−0.320	−0.485	−0.895	3.195
SB	0.118	0.077	0.146	0.002	0.188	0.188	0.021	0.037	0.141	0.213
P (regression)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Partial R^2 (regression)	0.19	0.11	0.21	0.04	0.28	0.23	0.34	0.32	0.10	0.34
Partial R^2 (species)	0.59	0.82	0.23	0.89	0.47	0.46	0.42	0.41	0.61	0.43
CMI weighing of $(X - X_{mean})/STD$[a]	+1	−1	−1	+1	+1	+1	−1	−1	−1	+1

DM = dry matter (g/kg fresh matter); ash (g/kg DM); CP = crude protein (g/kg DM); GE = gross energy (MJ/kg DM); ADFom = acid detergent fiber (g/kg DM); NDFom = neutral detergent fiber (g/kg DM); IVTD = *in vitro* true digestibility (g/kg DM); NDFD = *in vitro* neutral detergent fiber digestibility (g/kg NDF); DNDF = digestible NDF (g/kg DM); INDF = indigestible NDF (g/kg DM); R^2 = *r*-square of the linear model; VC = variation coefficient (=RMSE/Mean, %); RMSE = root-square mean square error; *B* = regression on *D*; SB = standard error of B; Partial R^2 (species) = *r*-square of the species factor; Different letters within a column indicate significant differences at P<0.05.

[a] Sign of the weight of each normal deviate in the crop maturity index (CMI). The CMI sum was again normalized and a +1 constant term was added to avoid too many negative values.

2.3. Statistical analyses

2.3.1. Characterization of the botanic species and allometry of the constituents on growth

Univariate fixed effect GLM models (SAS/STAT® 9.2. SAS Inst. Inc., Cary, NC) were used to test the species (S), and the ontogenic factor was considered as days after seeding (*D*) on the digestibility traits, the connected variables and the chemical analyses. The full model (1) was:

$$Y_{ij} = M + S_i + R \times D_{ij} + E_{ij} \quad (1)$$

where *Y*, variable of the *i*th species of the *j*th replicate; *M*, common average; *S*, effect of the *i*th species; *R*, common allometric regression of *D*; E_{ij}, error term.

A sub-model (2) only considered the species:

$$Y_{ij} = M + S_i + E_{ij} \quad (2)$$

where *M*, common average; *S*, effect of the *i*th species; E_{ij}, error term.

The relevance of the allometric regression factor, corrected for species effects, was then deducted as the difference between the full and the sub-model (2).

A further sub-model (3) only considered the regression factor:

$$Y_{ij} = M + R \times D_{ij} + E_{ij} \quad (3)$$

where *M*, common average; *R*, common allometric regression of *D*; E_{ij}, error term.

The relevance of the species, at parity of *D*, was then established as the difference between the full and the sub-model (3).

2.3.2. Crop maturity index

A crop maturity index (CMI) was formulated with a summative equation, where the ten variables were standardized, then weighted according to their ontogenic sense, namely as +1 if the regression of that variable (lating) on *D* was positive and as −1 if that variable (earlying) declined over time: low (or negative) values, which indicate a less mature status, are in fact opposed to a more mature status for high (positive) maturity values. Finally, the resulting sum of the ten scores was standardized to obtain a unity mean and unity standard deviation. The template in the last row of Table 2 suggests how to

calculate the CMI. The meaning of such a calculated trait, a normal distributed variable with 1 mean and 1 standard deviation, is that each value refers to the centroid of the ten variables of the 158 samples. A frequency of 15.86% of the CMI values could be expected below zero. Univariate fixed effect GLM models were applied to the CMI with a species classifier or with a family classifier. A correlation study pointed out the dependency of the CMI values on their raw components.

2.3.3. Prediction of digestibility and maturity from chemical constituents and correlations

The study concerning whether IVTD, NDFD and CMI could be predicted by chemical analyses was conducted without considering the species or the phenological factors. The chemical equations were obtained by regression of the predictand variable (Y) on all of the seven predictor variables (X):

$$Y_m = K + \sum_{1}^{7} \beta_i \times X_i + E_{ij} \quad (4)$$

where $m = 1$; Y_1 = IVTD; $m = 2$, Y_2 = NDFD; Y_3 = CMI; K = constant; β_i = ith regression coefficient for the ith X variable; E_{ij} = error term.

The z-score obtained from the Fisher transformation according to Preacher (2002) was utilized to compare the corrected R^2 coefficients.

A Pearson correlation coefficient table of the constituents, digestibility and CMI was calculated.

2.4. Vibrational spectroscopy and chemometrics

The Spectrum IdentiCheck™ FT-NIR/MIR system (PE, Perkin-Elmer, Beaconsfield, Buck, England) was used to scan the freeze-dried (FD) and oven-dried (OD) samples over a range from 714 to 3333 nm (2751 absorbance points); a glass Petri cup was filled with 8 mm of sample, then rotated in the dark, and scanned in duplicate for 30 repeated measurements. A portable LabSpec 4 Standard-Res Lab UV-Vis-NIR Analyzer fiber optic diode array spectrophotometer (ASD, Analytical Spectral Device Inc., Boulder, CO) was used to scan the same samples over a 350–1025 nm range (676 absorbance points); the scan was conducted in duplicate for 30 counts on the reversed plastic jar, whose top was protected by a plastic film, which was previously compensated for the white reference disk. The native spectra of the two instruments were averaged according to the sample, then processed, without any mathematical pretreatment, in order to enhance the fundamental vibrational properties, using WinISI II software (Infrasoft International, ISI: State College, PA, USA). The modified partial least squares (MPLS) method, permits one passage to eliminate outliers ($t > 2.5$). A cross-validation test with 4 groups was utilized to obtain the optimized equations for each considered variable. The prediction capacity of the calibrated models was then evaluated with the 1-VR parameter, which is routinely used by WinISI users and researchers (Nousiainen et al., 2004; Mentink et al., 2006), and with ratio-performance deviation (RPD; Williams, 1987; Williams and Sobering, 1996), a capacity parameter defined as the relationship between the standard deviation of the chemical method (SD reference) and the standard error in cross-validation mode (SECV) generated in the PLS model. When the RPD values were ≥2.5, the relevant calibration models were usually considered to be suitable for routine use. PLS offers more prediction stability than multiple linear regression, ridge regression or other well-known regression techniques (Höskuldsson, 1988). The PLS method is a chemo-metric tool that was not originally designed for statistical discrimination. However, applied scientists routinely use PLS for classification purposes (Fearn, 1997), and there is substantial empirical evidence to suggest that it performs well in that role. Barker and Rayens (2003) replaced heuristics with a formal statistical explanation, and it soon became clear that PLS is preferable to principal component analysis when discrimination is the goal and dimension reduction is needed. Since the two instruments work over very different spectral ranges, it is useful to subset the radiation in order to compare the performances of the instrument, as far as the method used for sample preparation is concerned. The spectra were thus studied over four bands; band-A (350–713 nm) was explored the UV–Vis in the ASD; band-B (714–1025 nm) was explored as NIR in the ASD instrument and as FT-NIR in the PE IdentiCheck; band-C (1026–2499 nm) was only explored as FT-NIR in the PE instrument; finally, band-D (2500–3333 nm) was explored as FT-MIR (medium infra-red) by means of the PE instrument.

The FD and the OD spectra were first considered separately, but the FD and OD spectra and equations were reciprocally validated. The two kinds of spectra were then merged in a combined set, in order to: (i) study the discriminative ability of the two preparates over all the spectral bands; (ii) obtain more powerful equations, provided a valuable reciprocal validation had been achieved; (iii) find a few dominant wavelengths in the spectral regions, using the stepwise regression method.

Four main factors were important in this experiment, namely the sample preparation, the two instruments, the spectral bands and the examined constituents and digestibility. The combinations of the instruments, preparations and spectral bands were elaborated as RPD values: the Friedman test (StatBox v. 1.5, Grimmersoft Logiciels, Paris), paired across the variables, was used to compare ten RPD sets of equations, derived from the full combinations of the results from the two instruments, the two preparations and the four spectral bands. Some partial linear combinations of the instruments, of the spectral bands, and of the preparations were also tested with this method. A comparison of the efficiency of vibrational spectroscopy was made between the different variables, which considered the 1-VR parameter of the PLS equations and the r^2v coefficients of the reciprocal validation of the preparate within variables; a z-score test, obtained from a Fisher transformation, according to Preacher (2002), was utilized for comparison purposes.

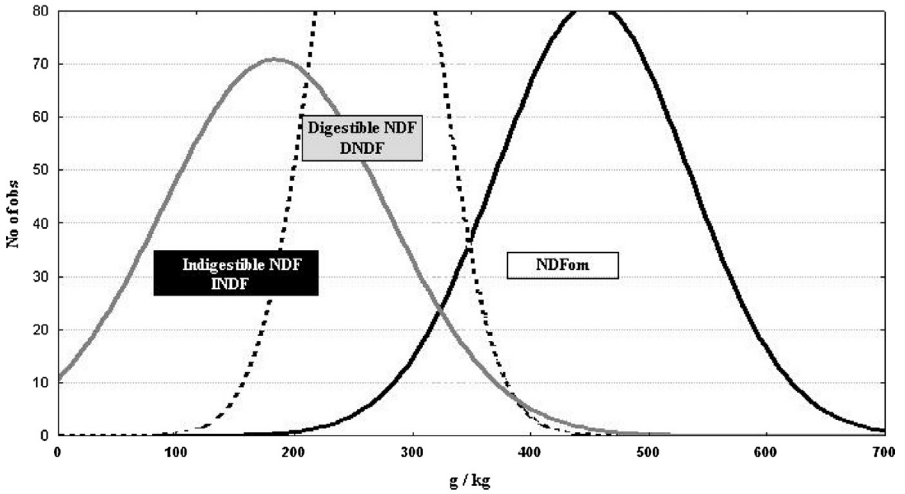

Fig. 1. Multiplot distributions of the indigestible neutral detergent fiber (INDF: 182 ± SD 94 g/kg; VC 52%), the digestible NDF (DNDF: 268 ± SD 49 g/kg; VC 18%) and their total (NDFom: 450 ± SD 82 g/kg; VC 18%). The DNDF appears to be the less variable trait since it has a narrower distribution than to the total NDFom (SD: −40%) and the indigestible part (SD: −48%). It should be noted that the indigestible-to-digestible NDF ratio is 0.68 for the means, but 1.91 for the standard deviation.

3. Results

3.1. Characteristics of the species and phenological effects

Table 2 reports the characteristics of the twelve crops that were analyzed by means of the linear models. The average *r*-square for species factor was 0.53, with the highest fit for GE (0.89), and the lowest fit for crude protein (CP, 0.23). On average, the *r*-square obtained for the regression on the days from seeding (*D*) was 0.22, but varied from 0.04 for GE to 0.32–0.34 for the digestibility parameters. In another model, which fitted individual regressions for each species, but which has not been reported here, it was established that the twelve species had specific linear coefficients, but in each case the regression coefficients pertinent to the species were all significantly positive or all significantly negative; thus, only the sign of the coefficient regression was considered for the calculation of the CMI. The significance of the regressions, connatural with the ontogenic process, lead to the exclusions of the isometric growth and the linear allometric pattern. Five constituents: DM, GE, NDFom, INDF and ADFom appeared to increase with the maturity stage. Conversely, the two digestibility traits and three constituents: ash, CP, and DNDF manifested a decreasing pattern, with negative coefficients on *D*. As can be seen in Table 2, a wide range of significant differences spread the species across all of the constituents and the digestibility features. The NDFom content deserves special mention. Fig. 1 highlights the distribution of the NDFom in its components. The smallest part was indigestible NDF (mean 182 g/kg), whose position in Fig. 1 appears shifted left, but it also features a similar extended distribution of the NDFom values. The largest part was digestible NDFom (mean 268 g/kg), a less variable constituent, which is characterized by a very narrow distribution, and is enhanced in Fig. 1.

3.2. CMI and indirect prediction of digestibility traits by means of chemical analyses

Table 3 reports the LsMeans for the CMI of the crops and the botanical families sorted according to increasing values. Several CMI gradients separated the twelve crops: flax (2.41), false flax (2.01), and ravizzone (1.73) registered the highest maturity values; hemp (1.22), galega (0.98), safflower (0.97), sunflower (0.74) and white lupin (0.74) were in the central position; chia (0.56), perilla (0.37), quinoa (0.08) and borage (−0.23) represented the least mature type. When the twelve crops were grouped according to their families, as highlighted in Fig. 2, six gradients differentiated their height families, namely from the least mature type: *Boraginaceae* and *Chenopodiaceae* < *Lamiaceae* < *Asteraceae* and *Fabaceae* < *Cannabaceae* < *Brassicaceae* < *Linaceae* (most mature type).

The indirect prediction of the digestibility parameters depends on the relationships between the variables (Table 4); all seven predictors were significant, and all of them were also significantly involved in the CMI prediction. The IVTD was accurately predicted (R^2 0.88) while the NDFD was less accurately estimated (0.78). As far as the CMI prediction is concerned, which is a salient feature of this paper, the closest fit (0.96) was observed when the seven chemical and phenological traits were used, with direct proportionality for *D*, DM, ADFom and GE, and inverse proportionality for ash, CP and NDFom. When the CMI variation was studied considering the digestibility features (Table 5), the most closely related components were identified as IVTD (r −0.97), NDFD (−0.93) and INDF (0.91); however, the CMI appeared to be closely related ($|r| > 0.50$) to all the other traits. The sign of the coefficients in the correlation table can be expected, because of the algorithm at the basis of the CMI. The last two rows report the coefficients of the regression equations of the CMI on each of the *X*-variables.

Table 3
LsMeans for the crop maturity index of the twelve crops and of the eight botanical families, sorted according to increasing values.

Species	Crop maturity index	Family	Crop maturity index
Borage	−0.23a	*Boraginaceae*	−0.23a
Quinoa	0.08ab	*Chenopodiaceae*	0.09a
Perilla	0.37c	*Lamiaceae*	0.47b
Chia	0.56c	*Asteraceae*	0.85c
White lupin	0.74cd	*Fabaceae*	0.89c
Sunflower	0.74cd	*Cannabaceae*	1.21d
Safflower	0.97de	*Brassicaceae*	1.88e
Galega	0.98de	*Linaceae*	2.41f
Hemp	1.22e		
Ravizzone	1.73fg		
False flax	2.01g		
Flax	2.41h		
R^2 (full-model 1)	0.82		0.81
VC %	44		45
RMSE	0.44		0.45
Mean	1.00		1.00
P (model 1)	<0.0001		<0.0001
B (regression)	+0.031		+0.030
SB	0.002		0.002
P (regression)	<0.0001		<0.0001
Partial R^2 (regression)	0.28		0.30
Partial R^2 (species)	0.54		0.51

R^2 = *r*-square of the linear model; VC = variation coefficient (%); RMSE = root-square mean square error; *B* = regression on days after seeding; SB = standard error of B; different letters between the crop maturity index (CMI) indicate significant differences within a column at P<0.05.

3.3. *Effect of the sample preparation and comparative efficiency of the spectral band of the two instruments*

The average absorbance spectra of the samples prepared by the OD or FD methods, and scanned by the PE or ASD instruments are reported in Fig. 4. A comparison of the instruments has highlighted that the OD samples are more radiation absorbent than the FD preparations. When comparing the common spectral B-band (714–1025 nm) with the other bands, a lower absorbance in the ASD instrument can be observed. Radiation is characterized by a sudden rise in absorbance after 2500 nm till 3333 nm, in the medium IR band.

As shown in Table 6, a very high discrimination was achieved in the C (RPD 7.86), A (5.37) and D (4.53) bands, while a minor but substantial discrimination was observed in B. The ASD and PE instruments basically appeared equivalent in the discrimination of the preparation. The comparative performances of the two instruments, synthesized in the RPD parameter, are reported in Table 6 separately for each preparation (FD *vs.* OD) and for each considered spectral band (A, B, C or D) of the two instruments (ASD and PE), for a total of 10 columns. The test on the RPD for the overall considered method of preparation was not significant (FD 2.47 *vs.* OD 2.03; P 0.08), but some interactions occurred, as can be seen from the fact that the average response differed in the instruments and in the spectral regions: the ASD instrument in the A-band did not perform significantly better for the FD samples *vs.* OD (RPD 1.94 *vs.* 1.79; P 0.25), while the OD preparation in the B-band was very efficient *vs.* FD (1.72 *vs.* 1.54; P 0.03). A comparison of the instruments on the pooled FD and OD preparations over the B-band was favorable for ASD *vs.* PE (1.63 *vs.* 1.21; P 0.0005). In the most usual NIR band, that is, the C-band, a significant superiority of the FD preparation was shown (2.46 *vs.* 1.95; P 0.004), while, unexpectedly, the MIR band, D, appeared 25%

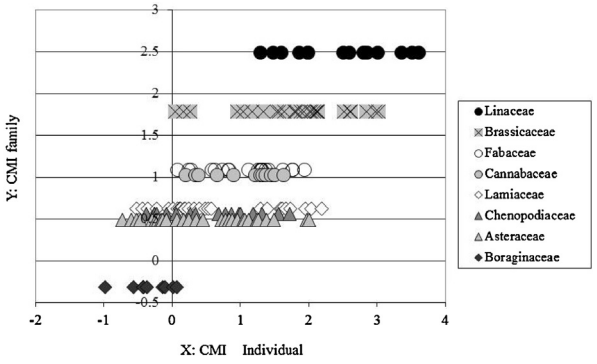

Fig. 2. Biplot distribution of the crop maturity index (CMI) over the families, from the least mature *Boraginaceae* to the most mature *Linaceae* (*X* axis: CMI of individual samples, *Y* axis: CMI means of the families).

Table 4
Prediction of digestibility traits and crop maturity index regressed on the chemical analyses.

Predictor variables	Mean	SD	IVTD		NDFD		CMI	
			Coeff ± std	P	Coeff ± std	P	Coeff ± std	P
			142.92 ± 13.34	0.00	154.46 ± 28.43	0.00	−5.2487 ± 0.793	0.00
D	611.8	19.94	−0.063 ± 0.017	0.00	−0.136 ± 0.036	0.00	0.00388 ± 0.001	0.00
DM	150.0	5.23	−0.067 ± 0.009	0.00	−0.141 ± 0.020	0.00	0.00639 ± 0.001	0.00
Ash	127.9	4.51	−0.028 ± 0.019	0.06	−0.061 ± 0.039	0.06	−0.00176 ± 0.001	0.06
CP	137.8	4.21	−0.004 ± 0.011	0.36	0.041 ± 0.024	0.05	−0.00202 ± 0.001	0.00
NDFom	450.2	8.19	−0.008 ± 0.007	0.13	0.068 ± 0.015	0.00	−0.00060 ± 0.000	0.08
ADFom	345.3	7.82	−0.064 ± 0.008	0.00	−0.115 ± 0.016	0.00	0.00514 ± 0.000	0.00
GE	16.70	1.12	−1.030 ± 0.761	0.09	−3.127 ± 1.622	0.03	0.24272 ± 0.045	0.00
Mean			81.7		61.4		1.00	
Total SD			9.4		14.7		1.00	
SE			3.27		7.0		0.19	
R^2 corr			0.88b		0.78c		0.96a	

Predictor variables: *D* = days after seeding; DM = dry matter (g/kg fresh matter); ash (g/kg DM); CP = crude protein (g/kg DM); NDFom = neutral detergent fiber (g/kg DM); ADFom = acid detergent fiber (g/kg DM); GE = gross energy (MJ/kg DM).
Predicted variables: IVTD = *in vitro* true digestibility (g/kg DM); NDFD = *in vitro* neutral detergent fiber digestibility (g/kg NDF); CMI = crop maturity index.
Coeff = regression coefficient; Std = standard deviation of the regression coefficient; SD = total standard deviation; SE = standard error of the regression; R^2 corr: corrected *r*-square of the regression model; different letters within the R^2 corr row indicate significant differences: a > b > c, P<0.05.

Table 5
Pearson correlation coefficients for the constituents, digestibility and crop maturity index (CMI), with regression coefficients of the *X*-variables on the CMI.

X-Variables	Ash	CP	NDFom	DNDF	INDF	ADFom	GE	IVTD	NDFD	*D*	DM	CMI
Ash	1	**0.31**	**−0.64**	**0.26**	**−0.70**	**−0.67**	**−0.90**	**0.67**	**0.64**	**−0.26**	**−0.71**	**−0.80**
CP		1	**−0.57**	0.09	**−0.40**	**−0.67**	−0.07	**0.55**	**0.54**	**−0.52**	**−0.38**	**−0.50**
NDFom			1	−0.02	**0.77**	**0.85**	**0.52**	**−0.85**	**−0.72**	**0.57**	**0.77**	**0.84**
DNDF				1	**−0.49**	**−0.28**	**−0.25**	**0.49**	**0.66**	**−0.20**	**−0.28**	**−0.47**
INDF					1	**0.80**	**0.64**	**−0.88**	**−0.84**	**0.68**	**0.77**	**0.91**
ADFom						1	**0.56**	**−0.88**	**−0.81**	**0.53**	**0.66**	**0.87**
GE							1	**−0.60**	**−0.57**	0.15	**0.65**	**0.73**
IVTD								1	**0.96**	**−0.60**	**−0.81**	**−0.97**
NDFD									1	**−0.57**	**−0.75**	**−0.93**
D										1	**0.44**	**0.54**
DM											1	**0.86**
Regression coefficient of CMI on X	−0.02	−0.01	0.01	−0.01	0.01	0.01	0.70	−0.10	−0.06			1
Constant term of the regression	3.34	2.67	−3.55	3.47	−0.85	−2.90	−10.65	9.30	4.84			

CP = crude protein; NDFom = neutral detergent fiber; DNDF = digestible NDF; INDF = indigestible NDF; ADFom = acid detergent fiber; GE = gross energy; IVTD = *in vitro* true digestibility; NDFD = *in vitro* neutral detergent fiber digestibility; *D* = days after seeding; DM = dry matter; CMI = crop maturity index.
The significant coefficients (P<0.05) are in bold.

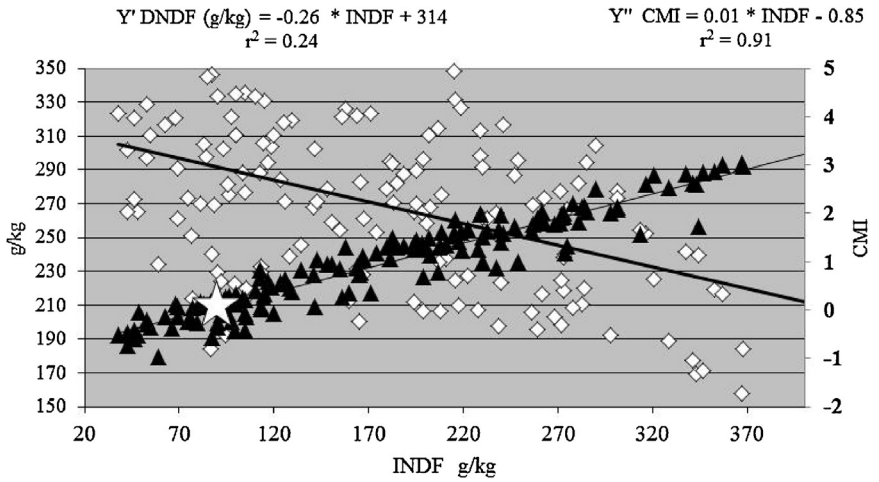

Fig. 3. Biplot of the indigestible neutral detergent fiber (INDF, g/kg) content on the crop maturity index (CMI, ▲; r^2 = 0.91) and on the digestible neutral detergent fiber (DNDF, g/kg, ◊; r^2 = 0.24). ☆ CMI predicted at 0.03 value from Nousiainen et al. (2004) data of INDF = 87 g/kg.

Table 6
Effect of the sample preparation and of the spectral bands on the ratio performance deviation (RPD, according Williams, 1987) of the two instruments, and discrimination of the two kinds of samples [freeze-dried (FD) *vs.* oven-dried (OD)] by means of vibrational spectroscopy in the different spectral bands.

Variables	FD					OD				
	ASD		PE			ASD		PE		
	A 350–713 nm	B 714–1025 nm	B 714–1025 nm	C 1026–2500 nm	D 2501–3333 nm	A 350–713 nm	B 714–1025 nm	B 714–1025 nm	C 1026–2500 nm	D 2501–3333 nm
Ash	2.01	1.68	1.15	3.3	3.39	2.05	1.62	1.34	2.39	3.44
CP	1.78	1.54	1.41	1.86	2.31	2.17	1.66	1.17	1.93	2.65
NDFom	2.08	1.6	1.19	2.53	3.15	1.76	2.04	1.37	2.09	2.33
DNDF	1.44	1.15	1.18	1.45	1.54	1.06	1.25	1.05	1.18	1.30
INDF	2.21	1.66	1.16	2.8	4.16	1.66	1.7	1.2	2.05	2.67
ADFom	2.05	1.45	1.15	2.43	3.11	2.42	1.9	1.05	2.27	2.79
GE	1.76	1.52	1.42	2.65	3.25	1.68	1.5	1.15	2.16	2.18
IVTD	2.22	1.58	1.15	2.81	4.30	1.72	1.69	1.19	1.97	2.67
NDFD	2.15	1.47	1.12	2.45	2.77	1.47	1.58	1.12	1.71	2.14
D	1.56	1.27	1.26	1.99	1.93	1.99	2.14	1.17	1.71	1.96
DM	1.88	1.71	1.25	2.25	2.80	1.71	1.71	1.32	1.81	2.40
CMI	2.19	1.84	1.15	3.01	4.18	1.8	1.9	1.28	2.07	3.11
Average columns	1.94	1.54	1.22	2.46	3.07	1.79	1.72	1.20	1.95	2.47
FT – Friedman's test	c	e	f	b	a	cd	d	f	c	ab
Average FD and OD columns			2.47					2.03		
Combined FD and OD set	ASD-A 350–713 nm		ASD-B 714–1025 nm		PE-B 714–1025 nm		PE-C 1026–2500 nm		PE-D 2501–3333 nm	
Average RPD	1.87 c		1.63d		1.21 e		2.21 b		2.78 a	
Freeze-dried *vs.* Oven-dried	5.37		2.70		1.98		7.86		4.53	

Variables: CP = crude protein; NDFom = neutral detergent fiber; DNDF = digestible NDF; INDF = indigestible NDF; ADFom = acid detergent fiber; GE = gross energy; IVTD = *in vitro* true digestibility; NDFD = *in vitro* neutral detergent fiber digestibility; *D* = days after seeding; DM = dry matter; CMI= crop maturity index.
RPD = Standard deviation/standard error in cross-validation; FT = Friedman's test differences of the columns within a row: a > b > c > d > e > f, P<0.05.

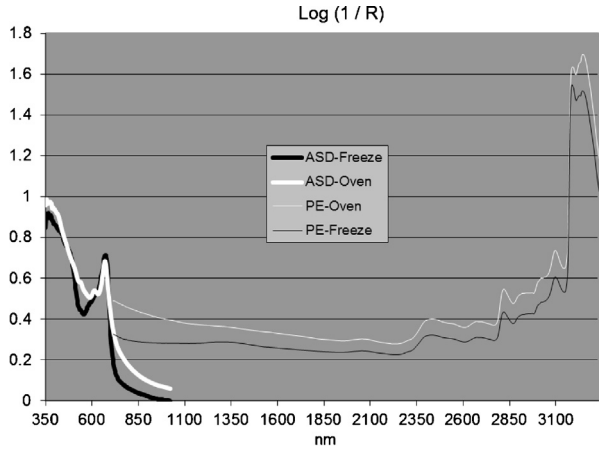

Fig. 4. Plot of the average absorbance spectra of the samples prepared by the oven-dried (oven) or freeze-dried (freeze) methods, and scanned by Perkin Elmer (PE) or Analytical Spectral Device (ASD) instruments.

more performing than the NIR band C (2.78 *vs.* 2.21; P 0.0005). It should be pointed out that the early NIR band, B, appeared rather inefficient, with the minimum RPD value (1.21).

3.4. Prediction of the chemical composition, digestibility and the CMI by means of vibrational spectroscopy

Table 8 reports the equations of the two instruments on the basis of the full spectral bands (AB for ASD and BCD for PE), but separately for the FD and the OD samples. The equations were also reciprocally validated over the 1-VR, *r*-square parameter of the internal cross-validation, and the r^2v parameter was then considered. If two equations of a constituent, calculated in the two preparations, were similar, then a successful reciprocal validation could be obtained. The results examined as 1-VR have shown that the sample preparation affected the prediction reliability. In general, the OD preparation was less efficient in the prediction of INDF, IVTD, NDFD and CMI in both instruments, while the DNDF and GE predictions were only decreased in the PE instrument. The *D* prediction increased in the ASD when the samples were oven-dried, while the NDFom prediction was unaffected by the sample preparation.

The ash content, the NDFom and its indigestible part, as well as the CMI were placed at the highest prediction rank in both instruments, albeit at different precision levels, on the basis of the instrumental capabilities, with an overall 1-VR avg. of 0.81. The worst performances were observed in the prediction of the DNDF, with an avg. 1-VR of 0.41. The brackets within these extremes distinguish different levels of efficiency in the predictions of the variables. The reciprocal validation depended mainly on the nature of the constituent and on the capabilities of the instrument. A significant bias (not shown in the table) was in fact always observed, because the spectra of FD and OD were very different; no mathematical pre-treatment was applied and the main bias depended on the different levels of absorbance in the two kinds of spectra. However, by ignoring the bias, a valuable verification of the correlation coefficient was achieved for both instruments in IVTD, INDF, ADFom, and in CP, NDFom for the ASD instrument, and in CPI, ash for the PE instrument. All these constituents, and also the NDFD, were

Table 7
PLS whole spectrum equations of the Analytical Spectral Device (ASD) and Perkin Elmer (PE) instruments for the combined freeze-dried (FD) and oven-dried (OD) samples, and two dominant wavelengths in the AB, BD and C bands.

Constituent	ASD instrument								Bands		PE instrument						Bands		
	Mean	SD	#o	RSQ	SECV	1-VR	# c	RPD	AB nm1	AB nm2	#o	RSQ	SECV	1-VR	# c	RPD	BD nm1	BD nm2	C nm
Ash	127.9	45.1	5	0.92	15.7	0.86	13	2.87	461	500	7	0.96	9.9	0.92	12	4.53	2408	2779	2451
CP	137.8	421	5	0.85	14.9	0.81	11	2.83	485	662	3	0.94	13.0	0.85	12	3.23	3307	725	2448
NDFom	450.2	81.9	4	0.90	26.8	0.86	11	3.06	698	998	5	0.91	27.0	0.84	13	3.03	2570	2559	2208
INDF	182.3	94.0	3	0.92	31.2	0.87	12	3.01	701	941	7	0.96	21.7	0.91	12	4.33	3330	848	2314
ADFom	345.3	78.2	5	0.84	30.3	0.81	11	2.58	656	659	5	0.96	19.5	0.90	13	4.02	3299	3170	2314
IVTD	817.5	94.0	3	0.86	35.8	0.83	12	2.63	701	941	7	0.96	21.5	0.91	12	4.38	3330	848	2314
NDFD	613.6	147.4	2	0.80	67.9	0.74	10	2.17	566	995	6	0.96	42.4	0.88	12	3.48	3330	720	2257
CMI	1.00	1.00	4	0.91	0.32	0.87	13	3.14	701	905	5	0.96	0.23	0.90	13	4.36	2799	2842	2451

Variables: Ash (g/kg DM); CP = crude protein (g/kg DM); NDFom = neutral detergent fiber (g/kg DM); INDF = indigestible NDF (g/kg DM); ADFom = acid detergent fiber (g/kg DM); IVTD = *in vitro* true digestibility (g/kg DM); NDFD = *in vitro* neutral detergent fiber digestibility (g/kg NDF); CMI = crop maturity index.

Mean = mean of the constituents in the whole dataset; SD = Standard deviation of the whole dataset; #0 = No. of outliers removed from the calibration; RSQ = *r*-square in calibration mode; SECV = standard error in cross-validation mode; 1-VR = 1-variance ratio; #c = No. of components in the PLS equation; RPD = relative prediction deviation (=SD/SECV); nm1, nm2 = first and second dominant wavelengths selected on the basis of a stepwise regression.

Table 8
Performances of the freeze-dried (FD) and oven-dried (OD) samples for the whole spectrum, with reciprocal validation, and differences in the predicted constituents, conducted separately for the Analytical Spectral Device (ASD) and Perkin Elmer (PE) instruments (1-VR values).

Variables	ASD instrument				PE instrument			
	Internal cross-validation		Reciprocal validation		Internal cross-validation		Reciprocal validation	
	FD 1-VR	OD 1-VR	FD-eq-OD r^2v	OD-eq-FD r^2v	FD 1-VR	OD 1-VR	FD-eq-OD r^2v	OD-eq-FD r^2v
Ash	0.70bc	0.70bc	0.04gf	0.00g	0.94a	0.92ab	0.71de	0.59ef
CP	0.69bc	0.80ab	0.56cd	0.73b	0.78de	0.75de	0.15hi	0.43g
NDFom	0.75b	0.74b	0.44de	0.64c	0.89bc	0.86bc	0.53fg	0.30gh
DDNF	0.42de	0.28e	0.10f	0.01g	0.61ef	0.23h	0.10i	0.00j
INDF	0.80ab	0.63c	0.49de	0.55cd	0.92ab	0.71de	0.56f	0.55fg
ADFom	0.74b	0.82a	0.46de	0.44de	0.87bc	0.79cd	0.48fg	0.57f
GE	0.67bc	0.57cd	0.00g	0.00g	0.90b	0.85c	0.20hi	0.36gh
IVTD	0.80ab	0.63c	0.50d	0.54cd	0.92ab	0.72de	0.64ef	0.55fg
NDFD	0.77ab	0.57cd	0.31e	0.45de	0.86bc	0.60ef	0.64ef	0.33gh
D	0.62c	0.74b	0.12f	0.34e	0.69e	0.70de	0.14hi	0.07i
DM	0.69bc	0.74b	0.12f	0.35e	0.87bc	0.79cd	0.28gh	0.63ef
CMI	0.82a	0.67bc	0.24ef	0.36e	0.94a	0.81cd	0.65ef	0.54fg

Variables: CP = crude protein; NDFom = neutral detergent fiber; DNDF = digestible NDF; INDF = indigestible NDF; ADFom = acid detergent fiber; GE = gross energy; IVTD = *in vitro* true digestibility; NDFD = *in vitro* neutral detergent fiber digestibility; *D* = days after seeding; DM = dry matter; CMI = crop maturity index.
FD-eq-OD = spectra freeze dried-equation-oven dried; OD-eq-FD = spectra oven dried-equation-freeze dried; 1-VR = R^2 in cross-validation; r^2v = *r*-square of the validation.
Different letters between all the 1-VR and r^2v coefficients within the instruments means significant differences: a > b > c > d > e > f > g > h, P<0.05.

calibrated and cross-validated in a combined FD-OD data-set (Table 7). The predictions were thus improved in both the ASD and the PE instruments, and reached avg. 1-VR levels of 0.83 and 0.89, with RPD of 2.8 and 3.9, pertaining to the 11.6 and 12.4 components in the PLS equations. In the combined set of samples, IVTD was predicted with a 1-VR of 0.83 and 0.91, and NDFD with a 1-VR of 0.74 and 0.88, by the ASD and PE instruments, respectively.

4. Discussion

4.1. Characteristics of the species and phenological effects

Čop et al. (2009) investigated five grasses (gen. *Alopecurus*, *Arrhenatherum*, *Dactylis*, *Lolium* and *Holcus*), and confirmed that a parabolic relationship of the CP concentration with time was typical for all of the five species; the NDFom and ADFom concentrations in the herbage of the five species differed above all during the mid-period of primary growth; their increases in time showed curvilinear (sigmoid and parabolic) relationships. The present work is based on four stages, which are not sufficient to interpolate a complete curve, but allows to describe an allometry of the chemical composition and of the nutritive value. However, the general trends were constantly confirmed in the covariation of the constituents and of the nutritional properties over a time series and across different species. Nousiainen et al. (2004) introduced the concept of bi-components in NDFom; in a study on grass silages, in analogy with Fig. 1, the standard deviations for INDF, DNDF and NDF were 33, 47 and 65 g/kg, respectively, while they measured 94, 49 and 82 g/kg in the present work, thus highlighting a similar variance for the digestible NDF, but a three-fold higher variability of the indigestible fraction, a result that is due to the wider spectrum of ontogenic stages and botanical resources introduced into the present study. It should be noted that the means of the three components INDF, DNF and NDFom in the grass silages of Nousiainen et al. (2004) were 87, 461 and 556 g/kg, respectively, a result that is quite different from the 182, 268 and 450 g/kg of the present work.

4.2. CMI and indirect prediction of digestibility traits by means of chemical analyses

In literature, attempts have usually been made to compare the variability in maturity of perennial forage grasses by describing and quantifying the growth stages. Moore et al. (1991) defined primary and secondary growth stages as well as their numerical indices, and provided descriptions to compare the stages of growth and development of perennial grasses. Numerical indices are useful to calculate various statistics in order to describe a population of grass tillers. Analogously, a decimal coding system was described and utilized for comparative purposes in cereals (Zadoks et al., 1974). Multispecies models have rarely been used for grasses. Nafus et al. (2009) predicted a current year's biomass for eight grass species common to semi-desert rangelands in the southwestern part of the United States. The CMI method presented in this paper applies to allometric compositional changes during ontogeny of the grass crops: it can be considered quite original for two reasons: (i) it is focused on the primary growth of a compound of species that represents families that are not usually harvested; (ii) it is a multivariate model that explores the within and the between-species ontogenic relationships, in terms of chemical composition and nutritive value of the crops. The relationship between digestibility and chemical composition is

very complex and depends on the botanical species (Bruinenberg et al., 2002). Moreover, predictive equations for digestibility exist in the literature, but despite their extensive use, evidence suggests that their application to poor quality forages has been relatively unsatisfactory or inconsistent between studies (Van Soest, 1994). NDFD is a predictor of forage digestibility that has been used for research purposes and routine forage analysis. Vendramini et al. (2010) noted a correlation between NDFD and IVTD in nine species and cultivars of warm-season grasses (r 0.88) which is similar to the 0.96 value observed in the present work. These close relationships are also caused by the automatic correlation of a part to the whole. Andrés et al. (2005), in a permanent meadow, reported a validated R^2 of 0.82 for DM digestibility using the Goering and Van Soest (1970) method, and observed that a summative equation, based on four chemical constituents, rose to R^2 0.87, a very similar value to the 0.88 observed in the present work in the Daisy IVTD. In this work, the digestible and the indigestible parts of the NDF appeared to be mildly antagonistic (r −0.49). The progressive and important modification of the NDF, from a digested form toward a non digested form, is clearly reflected in the CMI variation. As shown in Fig. 3, the combined CMI variable, that represents the aging process of the individual forage, appears to be positively close to the INDF (right Y axis; r^2 of 0.91), while the DNDF appears to decline as the INDF increases (left Y axis; r^2 of 0.24). The location of the estimated CMI in Fig. 3 at a value of 0.03 for the grass silages of Nousiainen et al. (2004), induced by an INDF of only 87 g/kg, is worth noting: those silages appear very immature compared to those of the present work; a similar check of the supposed CMI could be made using the other available X-variables, as indicated in Table 5. Attempts have already been made to classify crops as high-, moderate- or low-quality crops, on the basis of the nutritional value, *in primis* digestibility (Bruinenberg et al., 2002). However, the adopted separation into three categories of forage quality was arbitrary and the phenological evolution should be taken into account. In this paper, the incidence of the stage of maturity evolution (regression on D; Table 3) was evidenced by an r-square of 0.22, which was less than half the value found for the between-species variation (0.53); as a consequence, a large range of green and sub-mature crops was exploited, according to their ontogenic evolution, to build a robust calibration model. The proposed CMI has been adjusted for the phenological footprint and it has efficiently highlighted the botanical differences.

4.3. *Effect of the sample preparation and comparative efficiency of the spectral band of the two instruments*

Alomar et al. (1999) observed the same pattern in 20 samples of silage in an NIR range from 1100 to 2500 nm; instead, we have here considered the 350–713 nm visible band and the difference in absorbance has been confirmed. This could be due to several factors. The FD process, thanks to the sublimation of the ice crystals, allows a soft structure of the ground matrix to be maintained, while the warming process causes the tissue structure to collapse and increases the final mass density of the ground, thus promoting a higher absorbance of the matrix. With higher drying temperatures, or for long periods, some amino acids can give rise to Maillard products with sugars, or some proteins can be denatured and their solubility in neutral detergent reduced, thus increasing the NDFom and, eventually, the ADFom fraction. These alterations can increase the fiber content, and reduce the fermentation rate and extension of rumen inoculum, thus decreasing the *in vitro* digestibility of the organic matter and, hence, digestibility. Visible variations in the color properties of the two kinds of preparation are very common. The OD chemical transformation was not investigated in this work: in fact, the IVTD and NDFD were measured on the FD samples, while the constituents were determined on the OD preparations. However, the result of the perfect discrimination of the treatment over all the spectra obtained from the two instruments points out the presence of consistent physical and/or chemical factors. According Alomar et al. (1999), OD processing could decrease the CP level by about 5% (relative) and increase the fiber fractions by 1.3–2.3%.

The resulting poor resolution of all the variables in the first NIR band B confirmed the results of Park et al. (1997), who considered three wavelength ranges (400–2500 nm; 700–2500 nm; 1100–2500 nm) and, when predicting digestibility and intake, observed very little difference when the range was extended beyond 1100 nm. However, no reference is available about any remarkable advantage, as was instead observed in the present work, about the extension of the scan to the MIR region above 2500 nm. Clark and Lamb (1991) reviewed the selected NIRS wavelengths that are able to correlate DM digestibility; in a complete framework of 9 reviewed works and 57 reported nm (with R^2v of 0.83), two medians were featured at 1700 nm and 2300 nm. A concentration of the dominant wavelengths can be observed in some marginal bonds in Table 7; in fact, 12/16 were distinguished in the A band (<714 nm) for the ASD instrument, while 11/16 were identified in the MIR D band in the PE instrument (>2500 nm), and especially around 2900 nm. Nevertheless, after restriction of the calibration to the band-C, the 2314 nm wavelength was identified as the primary source for the ADFom contents, for the IVTD and also for the INDF. Moreover, 2451 nm was identified as the common primary source for ash and CMI variations. Finally, two particular wavelengths were related to CMI, namely 701 nm (red) and 905 nm (NIR). These wavelengths could be of future interest for a dynamic approach, what could involve crop ontogeny.

A critical point concerned the significantly low predictability of the digestible part of the NDFom, especially in the oven dried samples. A similar result has not been found in the literature. Nousiainen et al. (2004) observed that the cross-validation statistics were slightly higher for INDF (1-VR 0.91) than for DNDF (0.82). In the present case, the poor result may be ascribed to both the particular assortment of the samples, which showed a very narrow distribution of the digestible part of the DNDF (Fig. 1), but also to some denaturation of the DNDF during oven drying, probably due to the Maillard reaction and/or to a warm packing of the leaf, which in turn modified the optical properties of the most breakable part of the fiber, with depletion of the reflected vibrational signal. When the indirect prediction of the digestibility traits from the chemical analyses were compared with a prediction obtained by means of the NIRS method, somewhat surprising results were observed. According to Smith

et al. (1998), the NIRS prediction of digestibility in *Lolium rigidum* is better than indirect prediction *via* chemical analyses. In the present work, the equation obtained by means of vibrational spectroscopy in the more powerful PE instrument, and dividing the freeze-dried from the oven-dried spectra (Table 7), led to a very similar prediction of IVTD (1-VR 0.90) to the indirect combined chemical prediction (R^2c 0.88), while the rapid ASD, equipped with a limited UV–Vis–NIR and no FT instrument, was less efficient (1-VR of 0.74). Our results on IVTD are in agreement with those of Mentink et al. (2006), who observed a validated NIRS prediction of 0.85 in the total mixed rations, but our results are better than their 0.59 for NDFD.

Widening the field of observation to corn silage, a more mature fruited crop, and focusing on NDFD as the key parameter for high productive milking cows, Lundberg et al. (2004) found a poor prediction with NIRS, and stated that they hoped that NIRS calibration equations would be improved in the future. According to Mentink et al. (2006), NIRS is used in 60% of USA laboratories to predict feedstuff composition in the total mixed ration of dairy cows. NIRS calibrations for the prediction of NDFD on corn silage samples are available in some commercial forage testing laboratories. The variability involved in the data-set is very important, as Tran et al. (2010) showed in a consistent dataset of 2067 samples of milking cow diets, where a 0.96 R^2 cross-validated coefficient for DM digestibility was reported.

5. Conclusion

The vibrational spectroscopy of thermally prepared samples of crops harvested from the green to sub-mature stage can potentially be used to predict Daisy digestion parameters, with a precision that is almost equivalent to the prediction obtained from a regression based on any chemical parameter as the predictor. Freeze drying has been confirmed to be the gold standard, but oven drying could reach reliable prediction levels, except for the DNDF content. Rapid knowledge of the digestibility parameter of the total DM, and especially of forage NDFom, could suggest the optimum point for cutting and, after harvesting, its rational use in ruminant feeding.

The equations developed in the CMI framework cannot yet be extended to field applications, but since the vibrational spectroscopy tool has shown a remarkable estimation capability, this method, when applied to many oven and/or freeze-dried samples, has appeared reliable for a cheap and improved genetic evaluation in grasses. A substantial enlargement of the applied knowledge could be achieved by using vibrational spectroscopy directly on the intact part of forages. Four particular wavelengths have been identified as being related to CMI, namely: 701 (red), 905, 2451 and 2799 nm, respectively, in the A, B, C and D bands. Precision farming methods are currently undergoing unbridled development, and the use of optical sensors and resources are foreseen, but large-scale sampling field operations are absolutely necessary for the calibration of biomasses, and in particular of biomass constituents; however, this aspect was beyond the scope of this work.

Conflict of interest

None declared.

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