Quantification of Condensed Tannins in Sainfoin Powder (*Onobrychisviciifolia*) by FT-NIR Spectroscopy

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Abstract: Models based on FT-NIR spectroscopy and PLS-regressions were developed over three harvest years to determine the condensed tannins contents (CT, %(w/w)) in sainfoin powders. The three data sets corresponding to the three harvest years were used as calibration and then validation sets, successively. Finally, a global model gathering all the three years data sets has been developed.

The developed models predict CT in a range of 2.06 to 11.28 %(w/w). The accuracy of the models depended on the range of CT-values of the calibration and validation sets. Finally, it was possible to predict the CT with a SEP-value lower than 0.5% (w/w) and R^2-value higher than 0.9. In the present study, some of the PLS-parameters such as bias, slope and SEP have been statistically evaluated using the international standard ISO 12099:2017. The final global model was very promising since bias was not significantly different from 0, the slope was not significantly different from 1, and obtained SEP was 0.49% (w/w) while the calculated SEP-limit was 0.47% (w/w).

The presently developed model was robust over the three years and the global model presented very interesting values. Such approach would be very useful to develop a new quantitative, rapid and low cost method to assess the CT of sainfoin powders. This method will allow us to free ourselves from the traditional chemical method that consumes time, money and chemicals.

Keywords: Partial least square regression, principal component analysis, factorial discriminant analysis, ISO 12099:2017.

1. INTRODUCTION

The sainfoin (*Onobrychisviciifolia*Scop.) is a leguminous forage crop grown in Europe, Asia and western North America [1] whose nutritional qualities for livestock are recognized. This plant makes it possible to constitute a forage that is rich in proteins and that does not cause bloating. It is well known to be suitable to control gastrointestinal nematodes in small ruminants such as lambs [2, 3], ewes [4] or sheep [5]. Properties of sainfoin can also be combined with those of other plants to improve the ruminant digestion [6].

Currently, characterizing sainfoin and its condensed tannins contents by conventional methods (chromatographic) is time-consuming, expensive and requires several chemicals. In terms of speed of analysis, such method is difficult to use when a series of hundreds of samples have to be analyzed, as in the case of a breeding program.

Predictive, rapid and low-cost FT-NIR-based method could be an asset. Today, several agricultural products can be qualitatively and quantitatively characterized by FT-NIR spectroscopy [7-9]. Studies exploring the near- infrared spectroscopy to analyze the forage quality have been carried out. Most of them aimed at determining crude protein contents [10-12], cellulose intake or digestibility [10].The accuracy of NIRs-based models already published is about 1% (w/w) and R^2-value ranging from 0.80 to 0.95. To our knowledge, no publication concerning the prediction of condensed tannins on sainfoin has been carried out.

In the present study, a collection of three harvest years of sainfoin has been monitored in order to develop a FT-NIR-based model that could be able to predict the condensed tannins contents. Because the use of NIR spectroscopy in forage has to respect the EU standard “Animal feeding stuffs, cereals and milled cereal products -- Guidelines for the application of near infrared spectrometry”(ISO 12099:2017), the parameters of the PLS-models developed in the present study have been statistically evaluated.

2. MATERIALS AND METHODS

2.1. Plant Material

Three harvests of Sainfoin have been sampled for the present study (2014, 2015, and 2016). Plants were cultivated in Switzerland at the Agroscope (federal research institute for food and agriculture) (GPS: N46°12.724’ E7°18.201’), but also provided from on farm trials carried out in Switzerland and piloted by the Agroscope. A total of 340 samples were analysed to

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E-ISSN: 2409-9813/17

build the FT-NIR-based models. The sample collection contain plants gathered from: (1) two varieties ('Perly' and 'Perdrix'), (2) four planting densities, (3) four on-farm production sites, (4) Agroscope production sites, (5) four harvest stages.

2.2. Condensed Tannins Content

Condensed tannin content was analysed based on the method of [13]. Briefly, we suspended 50 mg of ground (0.5 mm) plant material with 5 ml of water in a 45 ml thick walled glass tube. Then we added 30 ml of n-butanol-HCL and gently swirled the glass tubes for 80 minutes in a water bath at 95°C. The samples were then immediately cooled down to 20°C. The supernatant was filtered through a 1.0 µm filter and then extinction was measured at 556 nm. Samples were analysed in duplicates and were compared to a purified standard of condensed sainfoin tannins. Procedure in brief as follows: we extracted the ground plant material with acetone/water (70:30 v/v). The extraction solution was exempted from acetone using the rotary evaporator, where water bath was not exceeding 38°C. Lipids and pigments were extracted from the remaining water phase with diethyl ether, and fibres were removed by centrifugation. The remaining extract was again concentrated by rotary evaporation. The condensed tannins were separated using the Sephadex LH-20-resin.

2.3. FT-NIR Spectroscopy

Spectra of Sainfoin powders were acquired in reflectance mode and direct contact analysis (DCA) using a FT-NIR spectrometer (MPA, Bruker). The analysis was performed in diffuse reflectance and using an integrating sphere. Absorbance spectra (average of 32 scans) were recorded at a resolution of 8 nm from 12000 cm^{-1} to 4000 cm^{-1}.

Powders were introduced in glass vials arranged on a sample rotator. The sample rotator makes it possible to rotate the sample on itself and the acquisition of 3 spectra per sample.

3. PLS MODELS

3.1. Chemometry

Spectra were gathered in a matrix $X_{n,p}$ where n is the number of spectra and p the number of wavelength steps. The reference-values (Condensed tannins content) were gathered in a column vector $y_{n,1}$.

The partial least square (PLS) models were elaborated in three steps: 1) determination of the optimal number of latent variables (LV) to be used in the final model, 2) calibration of the model, 3) validation with new data. Spectral and references data were gathered from 3 years of samples, 2 years samples were used to calibrate and the remaining year samples were used as external validation set. Then, the three years samples were gathered in a single data matrix and a global model was established using a cross-validation procedure.

The optimal number of LV has been achieved by using the minimisation of the root mean square error (RMSE).

Statistics and performances of models have been evaluated according to the coefficient of determination (R^2), the standard error of prediction (SEP), the bias and slope. The ratio performance to deviation (RPD) has also been calculated (WILLIAMS and SOBERING, 1993).

Unexplained error confidence limits(UECL) of SEP (eq. 1) are determined by performing a F-test (comparison of two variances) and calculating the T_{UE} (eq. 2) value. Such calculation allow to connect SEP with the SEC of the calibration model.

$$SEP = \sqrt{\frac{\sum_{i=1}^{n}(e_i - \bar{e})^2}{n-1}} \quad \text{eq. 1}$$

e_i : residual value; $\bar{e}$: bias; n: samples

$$T_{UE} = SEC * \sqrt{F(\propto, \vartheta, M)} \quad \text{eq. 2}$$

with $\vartheta = n-1$: Degree of freedom of the numerator associated with SEP of the validation set; $M = n_c - p - 1$: degree of freedom of the denominator associated with SEC; n_c: number of samples of the calibration set; p: number of dimensions of the PLS-model.

A bias (eq. 3) can be measured when a NIR-based model is established. Such systematic error can have many origins, for example when a sample is very different from the ones used for calibration, or when a NIR device drift occurred. Because many reasons could explain a bias in models, it is important to apply statistics to verify if the measured bias value is significantly different from0 or not. If the answer is positive, a bias-correction of the model can be carried out. To test the equality of bias-value = 0, a t-test is performed to determine the confidence limits of the bias-value (T_b) (eq. 4).

Table 1: Table of the Kruskal-Wallis ANOVA Applied to Condensed Tannins Contents. SS: Sum of Squares, df: Degree of Freedom, MS: Mean Squares, Chi-sq: Chi Square, Prob>Chi-sq: Probability Value

Source	SS	df	MS	Chi-sq	Prob>Chi-sq
Groups	$2.6*10^{7}$	2	$1.28*10^{7}$	407.02	$4.13*10^{-89}$
Error	$2.9*10^{7}$	865	$3.3*10^{4}$		
Total	$5.4*10^{7}$	867			

$$\bar{e} = \frac{1}{n}\sum_{i=1}^{n} e_i \tag{eq. 3}$$

with n: the number of samples; e_i : the residual of the ith sample.

$$T_b = \pm\left[t_{(1-\alpha/2)}.SEP\right]/\sqrt{n} \tag{eq. 4}$$

with α is the probability of type I error; t is the appropriate Student's t-value for a two-sided test with associated degrees of freedom SEP and the selected probability of a Type I error; n is the number of samples; SEP is the standard error of prediction.

The ideal slope of a NIR-based model is equal to 1. However, most models present slope-values slightly or strongly different from0. It is important to apply statistics to test if the slope-value is significantly different from0 or not. If the answer is positive, a slope-correction of the model can be carried out. To test the equality of slope-value = 1, a t-test is performed. The observed t-value is calculated (eq. 5) and compared to the t-value obtained from the t-test table ($t_{(1-\alpha/2)}$).

$$t_{obs} = |b-1|\sqrt{\frac{S_{\hat{y}}^2(n-1)}{S_{res}^2}} \tag{eq. 5}$$

With b is the slope; $S_{\hat{y}}^2$ the variance of predicted values; n the number of samples; S_{res} the residual standard deviation.

The slope will be considered as significantly different from1 if (eq. 6):

$$t_{obs} \geq t_{(1-\alpha/2)} \tag{eq. 6}$$

All data analyses were performed with OPUS software and Matlab R2017a.

4. RESULTS AND DISCUSSION

4.1. Variability of Condensed Tannins Contents

Sainfoin samples collected over the three years and over the growing conditions and sites constitute a data set from which the condensed tannins contents ranged from 2.06% (w/w) to 11.28% (w/w). The 2014 collection ranged from 2.68%(w/w) to 11.28%(w/w), the 2015 collection ranged from 2.06%(w/w) to 7.01%(w/w) and the 2016 collection ranged from 2.40%(w/w) to 7.68%(w/w). The data set of condensed tannins values, gathering over the three years, was not normally distributed. In order to compare the three data sets, a non-parametric analysis (Kruskal-Wallis) has been performed (Table **1**). The box plot (Figure **1**) highlights a significant difference between the 2014 data and the two other years data (2015-2016). However, the difference between years did not create separated clusters but a continuous range of values. Non-clustered data set is suitable to develop realistic linear regression models.

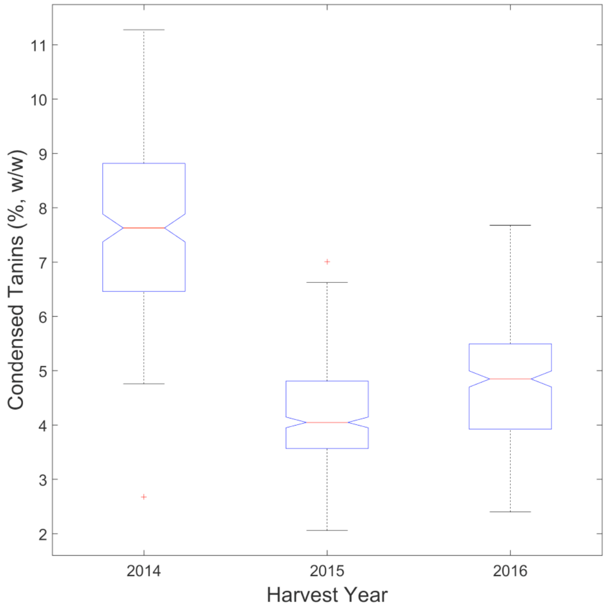

Figure 1: Distribution of condensed tannins according to the harvest years (2014, 2015 and 2016). Red line is the median-value, lower and upper limits of the whiskers are the 1st (25%) and the third (75%) quartiles, respectively. Data points beyond the whiskers are displayed using a red +. Lower and upper limits of the black dashed lines are the min and max values, respectively.

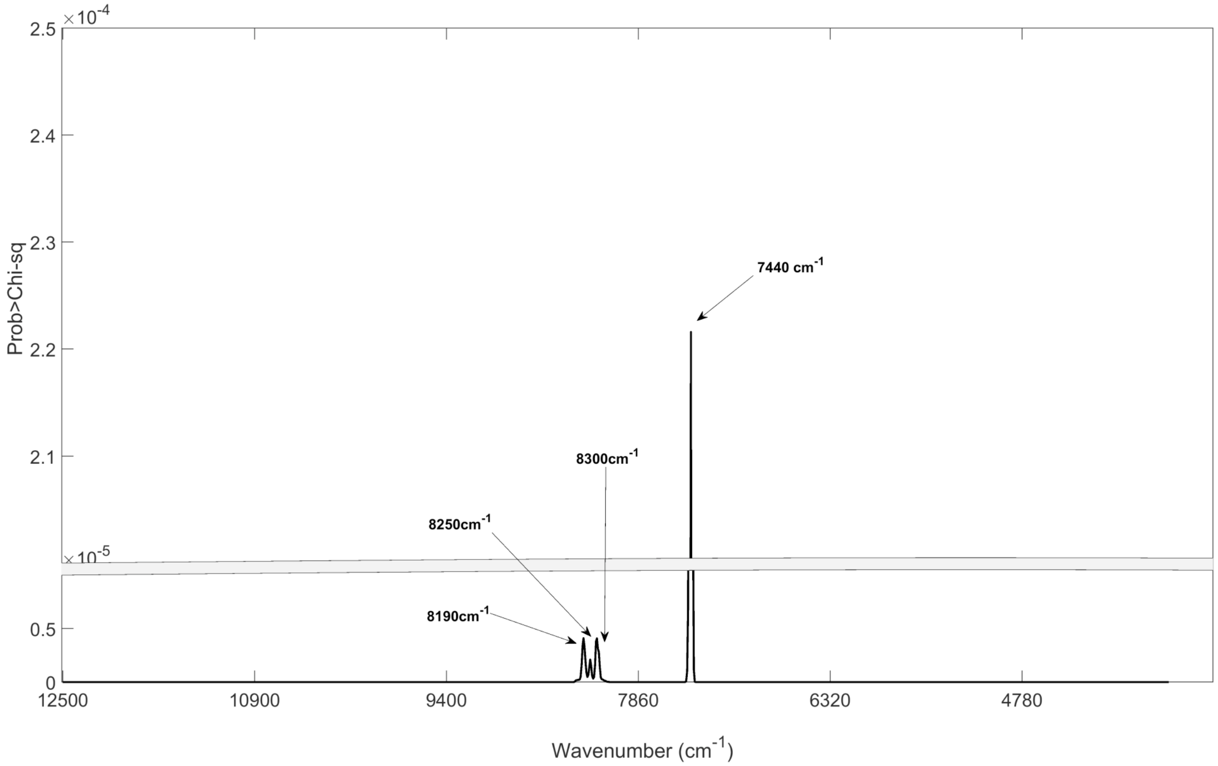

Figure 2: Analyses of Kruskal-Wallis processed on absorbencies of spectral data for each wave number.

4.2. Spectral Data

Series of Kruskal Wallis (KW) analyses of variance performed on three years spectral data are presented on figure **1**. The KW analyses aimed to test the differences between the harvest years, the years 2014, 2015 and 2016 counting 240, 489 and 291 spectra, respectively. The KW tests have been carried out on SNV-pre-treated spectra. P-values were under the 5% threshold for all wave number absorbencies (from 4000 to 12500 cm^{-1}). However, some of wave number absorbencies were less significant, these regions were in the vicinity of $8190cm^{-1}$, $8250cm^{-1}$, 8300 $cm^{-1}$and 7440 cm^{-1}.

A Principal Component Analysis (PCA) has been performed on spectral data to find potential outliers and to visualize the variability of the data set. The factorial map according to the first two principal components (PC) is presented on figure **3A**. The factorial scores are organized in a wide cloud without any evident outlier. The first PC (82%) explains the variability inside the sub-data sets of the harvest years, while the second PC (11.4%) describes a difference between the harvest years (figure **3B**). The first PC relies on absorbencies in the 4000 to $8200cm^{-1}$ and 10000 to $12500cm^{-1}$regions. The second one relies on the region comprised between 7600 and $8700cm^{-1}$.Absorbencies in the vicinity of $8670cm^{-1}$ is only relevant for the second PC.

Differences between harvest years is characterized by a PC explaining only 11.4%, however in order to analyse more accurately such differences, a modified factorial discriminant analysis (FDA)[14] has been performed on spectral data to discriminate between the harvest years and identifying the spectral region discriminating specifically the harvest years. The spectral data have been gathered in a rectangular matrix $X_{n,p}$ were n (n = 1020) is the number of spectra and p (p = 1050) the number of wave number steps. The [2n/3] spectra has been paced in a calibration set and the remaining [n/3] spectra in a validation set. In a first step, the optimal number of factorial scores has been determined for each spectral collection. To assess this optimal number, a FDA has been performed using all factorial scores available. Such an approach creates a voluntary over-fitting phenomenon. Then, the percent of individuals correctly classified in FDA (the harvest years) has been expressed as a function of the factorial score introduced. Finally, 10 dimensions have been selected to FDA applied to harvest year's discriminations. Factorial maps of the FDA according to the first factorial scores are presented in the figure **3C**. The factorial scores of harvest years are clearly gathered in three distinct

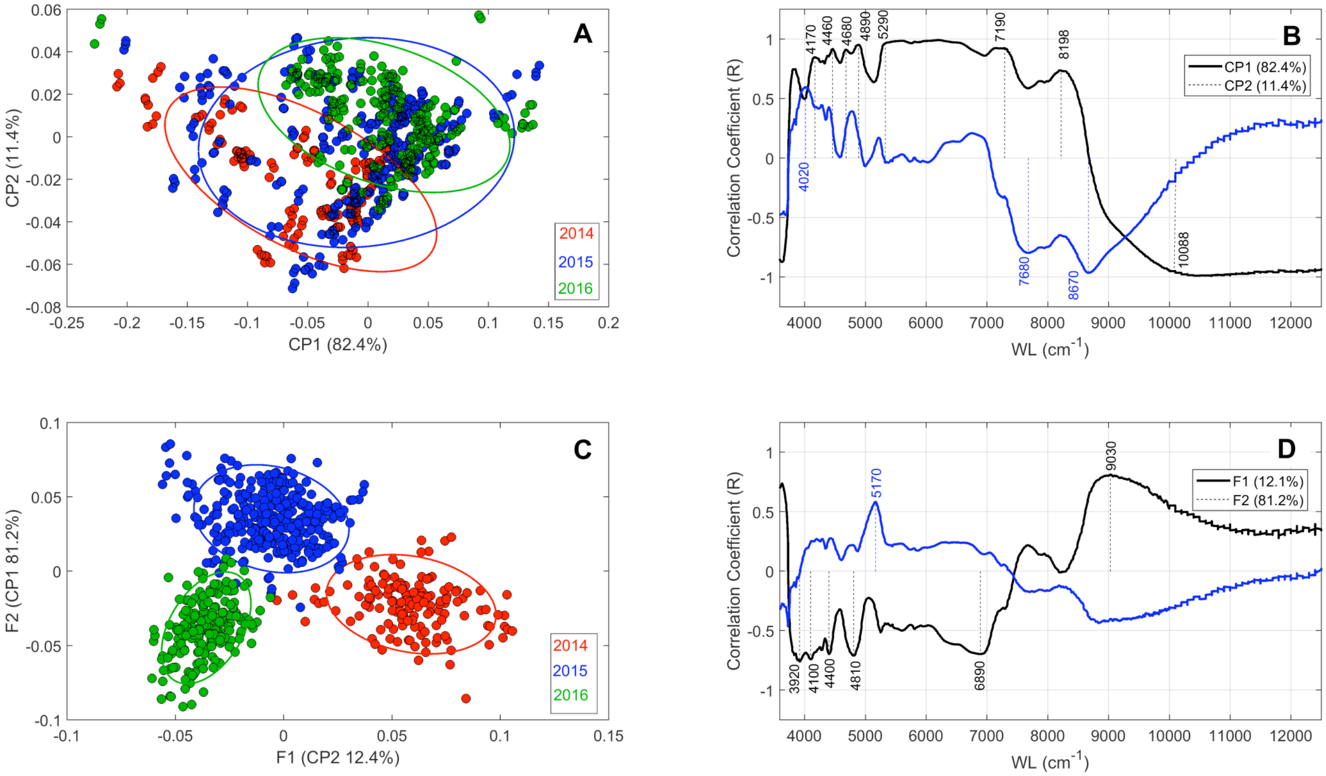

Figure 3: PCA-map according to the first 2 principal components (**A**), Correlation between the first two factorial scores (PC and PC2) of PCA with spectra absorbencies (**B**), FDA-map according to the first two factorial scores (**C**), Correlation between the first factorial scores of FDA (F1 and F2) and the spectra absorbencies (**D**).

batches. The percent of the samples correctly classified reached more than 98%. The factorial score (F1) explains the variability between the three sets corresponding to the three harvest years while the second factorial score (F2) explains some differences between the harvest year 2015 and the two others harvest ones 2014 and 2016 (figure **3D**). F1 is correlated to absorbencies between 3900 and 7000cm^{-1} (as in PCA) but also to absorbencies in the vicinity of 9000cm^{-1}. In PCA absorbency at 9000cm^{-1} was very low and not relevant to explain any variability. Similarly to the KW analyses (figure **2**), FDA (figure **3D**) highlighted that spectral region between 7400-8300did not have any relevance to classify the samples according to the harvest years.

4.3. PLS-Modelling

4.3.1. Modelling Based on Harvest Years

PLS models have been performed to predict the condensed tannins values in Sainfoin powder. Three models were attempted by using data of [2014-15], [2014-16] and [2015-16] as calibration sets, successively. Each model has been validated by using data of [2016], [2015] and [2014] as validation sets, successively. All three datasets were independently collected and analysed, allowing external validation of established models.

The PLS-values and statistics of models are presented in the table **1**. Models calibrated with [2014-15] and [2014-16] data present correct PLS-values. R^2-values are comprised between0.73 and 0.95, and RMSE is not higher than 0.54% (w/w). Furthermore, the bias-values are very low, in the order of 10^{-5} % (w/w). The validations of these two models have been carried out using the spectral data of [2016] and [2015], respectively. The ranges of CT-values of the validation sets were comprised in the ranges of those used in the calibration sets. The obtained R^2-values were 0.73 and 0.83, while SEP-values were 0.54% and 0.43% (w/w). The ratio performance to deviation has been calculated for both models and values of 1.89 and 2.26 have been obtained, respectively.

The model based on calibration [2015-2016] and validation [2014] partially failed. Although the R^2-values are correct, the SEP value increased from 0.44% (w / w) to 0.8% (w/w) between the calibration and the validation steps.

In order to evaluate bias, SEP and slope parameters, statistics have been applied. Firstly, bias-value has been evaluated by determining the confidence limits of the bias-value noticed "Tb". Bias-value (1.25 10^{-2}% (w/w)) of the [2014-15] calibration model respected the Tb limit (6.45 10^{-2}% (w/w)). Concerning this first model, we can assume that the bias-value is not different from 0. The [2014-16] calibration model is very close with respect to the bias-value limit, with a bias-value of 4.98 10^{-2}%(w/w) and a bias-limit of 4.28 10^{-2}%(w/w). The [2015-16] calibration model failed and the bias-value (5.99 10^{-2}%(w/w)) is about 6 fold higher than the bias-limit (1.10 10^{-2}%(w/w)).

T_{UE}-value is the SEP-limit and allow to evaluate the validation of a model. SEP of [2014-15] and [2014-16] calibration models respect or are very close to the SEP limit. SEP of [2014-16] model is about 25% under the limit, while SEP of [2014-15] model is just 8% upper the SEP limit. Concerning the [2015-16] model, SEP is 66% upper the SEP-limit.

The slope of the models has been statistically evaluated. Since the observed t-value is lower than $t_{(\alpha-\alpha/2)}$, the slope is not different from 1. All tested models passed the test and all slopes were not different from 1.

The figure **4** reports the actual values of CT as a function of the PLS-predicted values of TC. Figure 4A and 4B confirm the correct fitting of values of the first two models (2014-15 and 2014-16). The figure 4C explains the reason why the [2015-16] model failed. The main reason is that the range of TC-value of the validation set [2014] is out of the range of TC-values of

Table 2: PLS-Parameters and Statistics for Bias, SEP and Slope of each Model

	Harvest years							
	2014-2015	**2016**	**2014-2016**	**2015**	**2015-2016**	**2014**	**2014-2015-2016**	
PLS-parameters	**CAL**	**VAL**	**CAL**	**VAL**	**CAL**	**VAL**	**CAL**	**VAL**
n (spectra)	**595**	**275**	**481**	**386**	**658**	**207**	**870**	**870**
Spectra pre-treatment	**MSC**		**Normalization Min-Max**		**First derivative (17) + MSC**		**SNV**	
n (Samples)	198	92	160	129	219	69	290	290
min (%,w/w)	2.06	2.40	2.06	2.06	2.06	2.68	2.06	2.06
max (%,w/w)	11.28	7.68	11.28	7.01	7.68	11.28	11.28	11.28
Mean	5.45	4.75	6.00	4.26	4.46	7.66	5.23	5.23
SD	2.04	1.03	1.96	0.97	1.02	1.65	1.82	1.82
λ(cm^{-1})	7505.9 - 5446.2		6102-4242.8		9403.7-4597.7		9403.7-4242.8	
LV	9		6		7		10	
R^2	0.95	0.73	0.93	0.83	0.82	0.84	0.94	0.93
RMSE	0.46	0.54	0.53	0.43	0.44	0.80	0.45	0.49
RPD	4.29	1.89	3.58	2.26	2.10	2.05	3.93	4.23
Bias	-2.69E-05	1.25E-02	-1.04E-05	4.98E-02	1.52E-05	5.99E-01	1.82E-04	4.12E-03
Tb		6.45E-02		4.28E-02		1.10E-01		3.27E-02
		PASS		FAIL		FAIL		PASS
SEP		0.54		0.43		0.80		0.49
Tue		0.50		0.57		0.48		0.47
SEP limit		FAIL		PASS		FAIL		FAIL
Slope		0.91		0.84		1.41		1.00
$t(\alpha-\alpha/2)$		1.97		1.97		1.97		1.96
tobs		0.13		0.36		0.38		0.00
tobs - $t(\alpha-\alpha/2)$		PASS		PASS		PASS		PASS

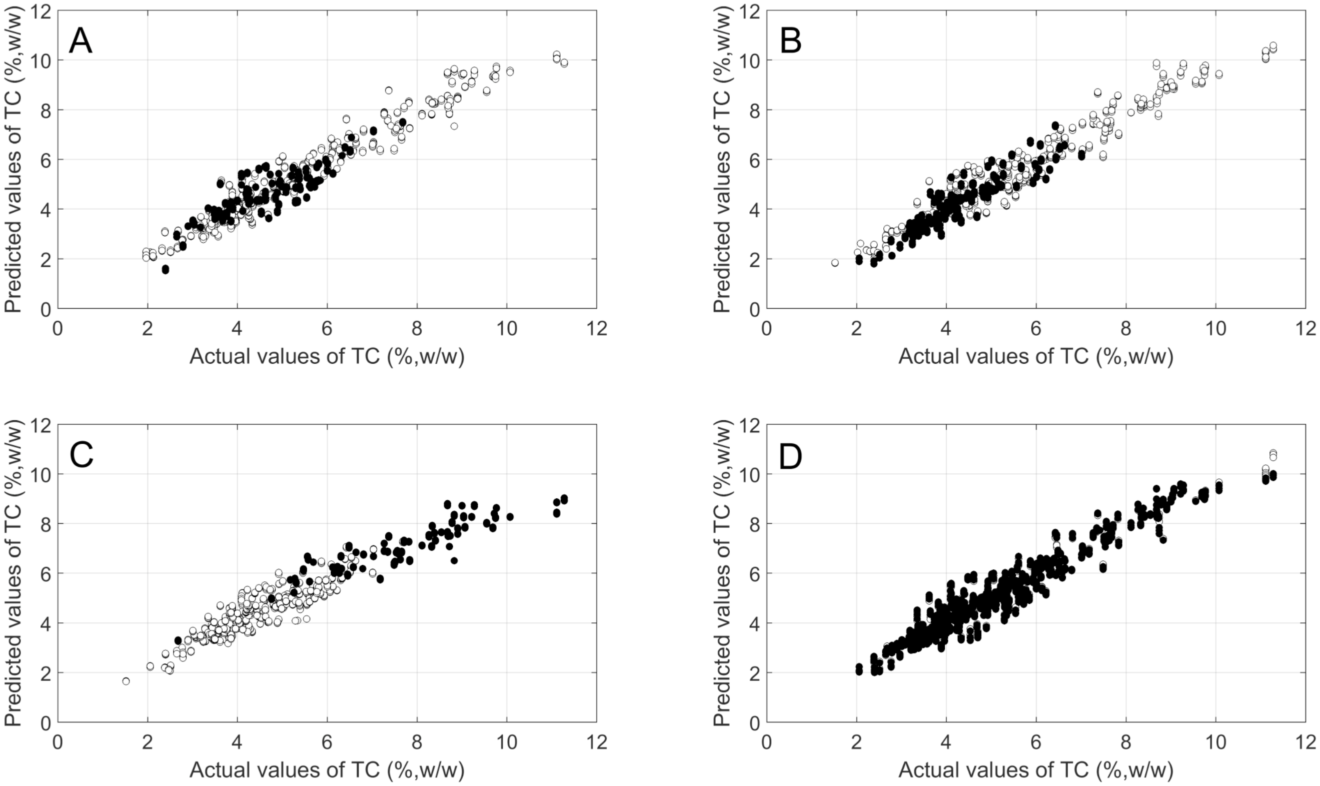

Figure 4: Actual *vs* Predicted TC-values by PLS modelling. (**A**): model calibration [2014-15] *vs* validation [2016], (**B**): model calibration [2014-16] *vs* validation [2015], (**C**): model calibration [2015-16] *vs* validation [2014], (**D**): Global model with “leave-k-out” cross-validation [2014-15-16]. White symbols represent calibration and black symbols represent the validation.

the calibration set [2015-16]. The higher value in calibration set is 7.68% (w/w) where the higher value of the validation set is 11.28% (w/w). Thus, all predicted values beyond 7.68% (w/w) tend to underestimate TC values resulting in abnormal high SEP-value in validation.

4.3.2. Global Three Years Model

Then, a global data set gathering on one hand the spectral data and on the other hand the TC-values of the three harvest years has been carried out. These two sets have been used to establish a global model aiming at predicting the TC-value by using spectral data.

This global model has been cross-validated by using a full “leave-k-out” procedure, where k=3 since three spectra were acquired per sample of powder Sainfoin. The actual versus predicted TC-values have been plotted in the figure 4D. In the present model, 870 spectra were used and PLS-values presented in the table 2 are promising. The model is correct in term of R^2 which are comprised between 0.93 (cross-validation) and 0.94 (calibration). SEP-value remained low at 0.49 %(w/w). Statistics calculated to evaluate some of PLS-parameters are also improved compared to previous calibrations. Bias-value is lower than T_b-value, and Slope is not significantly different from 1 ($t_{observed} < t_{(\alpha-\alpha/2),}$). Finally, evaluation of SEP is very promising since a value of 0.49%(w/w) has to be compared to a SEP-limit established to 0.47%(w/w). The global and final PLS model aiming at to predict the CT of Sainfoin powder by FT-NIR spectroscopy is promising and respect most of statistical evaluations. Furthermore, the wide range TC-values obtained by gathering the three years data sets allowed to obtain a good ratio performance to deviation in the vicinity of 4.23.

Near-infrared spectroscopy has already been applied to forage quality determination. Most of the studies evaluated the near-infrared spectroscopy for the determination of crude protein content (CP) or to predict the organic matter (OMD). Less than ten years ago, Decruyenaere, Lecomte (10) elaborated NIR-based calibrations to determine the CP, cellulose and OMD contents in forages. The accuracy of the models were characterized by R^2-values comprised between 0.88 and 0.95, SECV in the vicinity of 1% (w/w) and RPD between 1 and 4. In the present study, models aiming at predicting condensed tannins in sainfoin presented R^2-, SEP and RPD-values of 0.93, 0.49 % (w/w) and 4.23, respectively. In term of R2-value, the obtained are comparable to those of Decruyenaere *et al*. (2009) and they are higher in terms of SEP and RPD. Furthermore, the present results showed that an external validation of model guaranteed a SEP-value in the vicinity of 0.5 % (w/w).

As far as we know, there are no published study aiming at predicting condensed tannins from sainfoin by using FT-NIR spectroscopy. However, Dykes, Hoffmann (15) reported models predicting condensed tannins in sorghum. The authors found a correlation between NIR-spectral-data of R=0.82. The level of our model is higher although it is difficult to compare results from different plant materials.

CONCLUSION

The present study aimed at evaluating the FT-NIR spectroscopy coupled with chemometric modelling as a potential new method to determine the condensed tannins of sainfoin powder.

The study carried out to reach our objectives has been conducted over three years during which sainfoin samples have been harvested and condensed tannins quantified.

The established models presented satisfying accuracies and quite robust statistics. Models were validated by external validations. Condensed tannins can be determined with a standard error of prediction lower than 0.5 % (w/w), a R^2-value higher than 0.9 and a RPD-value higher than 4.

The PLS-parameters were correctly evaluated according to the international standard ISO 12099:2017. This standard is available for the use of near-infrared spectroscopy applied to “substance or product, including additives, whether processed, partially processed or unprocessed, intended to be used for oral feeding to animals”. Such standard has been validated by several countries such as: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, Former Yugoslav Republic of Macedonia, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and United Kingdom. Such statistics allowed to test the bias, the slope and the SEP-values obtained during validation models.

The results obtained in this study are crucial because they will allow our screening program to create forage of higher nutritional value. Until today, condensed tannins analyses were carried out by chemical analyses that took a long time, consumed chemicals and consumed money. In a close future, our screening programs will be able to use this new method, based on near-infrared spectroscopy, which will be fast, inexpensive and chemical free.

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Received on 19-12-2017 Accepted on 24-12-2017 Published on 29-12-2017

DOI: http://dx.doi.org/10.15377/2409-9813.2017.04.7