Using Attenuated-total-reflection Fourier-transformed spectroscopy to reveal molecular structural differences among willow (*Salix spp*.) foliage cultivars in relation to their potential as fodders.

Running title: Molecular structure of willow foliage cultivars

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ABSTRACT

Background: Willow trees represent a suitable species for the development of agroforestry systems integrating bioenergy and animal feed production. However, there is a lack of information regarding the suitability of leaves and stems, considered a bioenergy by-product, as animal feed. The aim of thist study was the employment of attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy (550-4000 cm^{-1}) to investigate differences in the nutrient molecular structure profile of leaves and stems of selected willow cultivars to understand their utility for ruminant nutrition.

Results: Univariate (ANOVA) analysis of leaves showed lower intensities of cellulosic compounds and higher of protein in comparison to stems, which suggests higher leaf dry matter and protein digestibility. Spectral analyses revealed differences in both plant parts between *Salix* "Terra Nova" cultivar and *S.* "Beagle", "Resolution" and "Olof". The main differences included higher α-helix to β-sheet ratio, which is related to higher protein digestibility and lower intensity of the spectral peaks located within the structural carbohydrate region (STC4), in correlation with lower content of condensed tannins. Principal Component and Agglomerative Hierarchical Cluster Analysis showed significant discrimination among willow cultivars in the cellulosic, STC and amide region, whereas differences were less evident for total carbohydrate and lipid-related regions.

Conclusion: The application of ATR-FTIR molecular spectroscopy is an effective tool to rapidly identify spectral features related to the nutritional composition of willow foliage and to discriminate between cultivars and parts of the plant. This information would be useful to optimise the use of willow fodders in agroforestry systems.

Keywords: willow; ATR-FTIR; agroforestry; molecular structure; ruminant nutrition; multivariate analysis

1. Introduction

The increasing food-feed-fuel competition for arable land in a climate change scenario urges to find more sustainable feeding strategies for ruminant-based food production, including the use of alternative and novel resources. Agroforestry, the integration of trees and agriculture, is valued as a multifunctional land use approach that balances the production of commodities (food, feed, fuel, fibre) with non-commodity outputs, such as environmental protection and soil conservation.[1] Short rotation woody crops (SRC) are fast-growing tree species cultivated to produce high biomass yields in a short period that can be used for energy purposes, being willow (*Salix spp.*) the most spread species in Europe.[2] Willow's leaves and stems up to 18 mm diameter are considered as bioenergy waste, thus representing a potential fodder for ruminants. Indeed, willow foliage shows excellent potential in animal nutrition due to higher storage of foliage trace elements than pasture species, a suitable content of crude protein(with values above 20%), and a relevant content in condensed tannins (CT). The consumption of CT by ruminant livestock has been related with the reduction of bloat incidence, antiparasitic properties, improvement of nitrogen utilization and reduction of greenhouse gas emissions.[3,4]

In the last years, willow genetic improvement programmes in Sweden and the United Kingdom (UK) made significant progress in breeding willow as SRC used for bioenergy, resulting in the development of cultivars that are more productive, more resistant against pests and diseases, and with stable yield levels.[5] However, data regarding differences in the nutritive value between SRC willow cultivars is scarce, thus hampering its efficient utilisation in animal diets. Besides species and cultivars, the nutritional composition and digestibility of willow, as other forages, may vary depending on the plant part, growth stage and environmental factors.[6] Therefore, the development of techniques that allow quick screening of the nutritional quality of foliage samples is essential to ensure efficient management of forages.

Traditional "wet" chemistry analyses employed to study the nutritional value of feeds are generally time-consuming, often require the use of extensive sample pre-processing and toxic solvents and, in addition, offer incomplete information, as they imply the sample breakdown and loss of three-dimensional structure. Attenuated total reflectance Fourier-Transformed Mid-Infrared spectroscopy (ATR-FTIR) is based on the absorption of an infrared (IR) evanescent wave travelling through a high refractive index prismatic crystal in close contact with a sample. ATR-FTIR is a rapid, non-invasive and non-destructive bioanalytical technique, which requires little amounts of samples. Therefore, it may represent an alternative to classical analytical methods for qualitative and quantitative analyses of large numbers of plant samples regularly. Besides, the data obtained is closely correlated to the

vibrational intensities of the molecular bonds of chemical functional groups of samples,[7] which are closely related to feed's quality and nutritive value for animals' diets.[8] However, up to date, there is no information regarding the nutrient molecular structure of willow foliage cultivars.

The number of applications of ATR-FTIR in the agricultural and animal nutrition field is increasingly growing. Indeed, it is considered the most accessible to study the secondary structure of proteins,[9] and has successfully predicted the protein degradation behaviour of different feeds and forages, such as sainfoin,[10] dried distillers grains,[11] or canola.[12] ATR-FTIR combined with multivariate statistical analysis, has been successfully applied to the differentiation and classification of *Citrus* species[15] and almond varieties.[16] More recently, this technique has been used to detect changes in the molecular structure of alfalfa induced by genetic modifications,[7,13] which can efficiently predict the ruminal fermentation kinetics.[14] Therefore, the application of ATR-FTIR to willow foliage samples might be useful to obtain an in-depth knowledge of the differential molecular profile of the plant parts and cultivars, thus combining the benefits of bioenergy production and animal nutrition for developing efficient agroforestry systems.

This research study aims to employ ATR-FTIR to screen the nutrient molecular structural differences between stems and leaves of the most common willow cultivars ("Beagle", "Olof", "Resolution" and "Terra Nova") employed as SRC for biomass production. Univariate structural features in protein, carbohydrate and lipid-related regions were assessed, as well as two multivariate analyses, agglomerative hierarchical cluster analysis (AHCA) and principal component analysis (PCA) were used to compare all spectral structural regions.

2. MATERIALS AND METHODS

2.1 Plant material

Four willow cultivars developed by crossing for bioenergy plantations, (Salix "Beagle", S. "Olof", S. "Resolution" and S. "Terra Nova")[17] were used in this study. Willow samples were collected from the experimental location at the Agri-Food and Bioscience Institutes (AFBI) Research Station in Loughgall, Co. Armagh, Northern Ireland (UK (54 o41'N, 6 o60'W). Whole branches were harvested from each of the four cultivars from each of the three randomly allocated, and previously tagged, trees from within each of the three complete replications in May in 2016 and 2017. The branches were further separated by hand into leaves and stems resulting in a total of 144 samples (4 cultivars x 2 foliage parts x 3 different trees x 3 replications x 2 years). Stems were up to 8 mm in diameter and 30 cm long as cattle have been shown to eat willow of 4-8 mm diameter[18]. Willow samples were lyophilised, ground and passed through a 0.02 mm (mesh 70) sieve (Glenammer Sieves Ltd., UK).

2.2 Nutritional composition

Dry matter (DM) (930.15), ash (942.05) and crude protein (CP) (Leco Protein/NAnalyser FP-528, Leco Corp., St Joseph, MI, USA) contents were measured according to AOAC (1990). Neutral detergent fiber (aNDF) (with sodium sulfite and heat stable alpha amylase) and acid detergent fiber (ADF) were determined sequentially according to Van Soest et al., (1991) and expressed including residual ash. Acid Detergent Lignin (ADL) was analysed from the ADF residue by digestion with 72% sulfuric acid for 3h (Van Soest et al., 1991). Condensed tannins were analysed by HCl-Butanol method (Reed, 1986).

2.3 ATR-FTIR Data and Collection Analysis

Molecular spectroscopic experiments were conducted at the Institute for the Global Food Security, Queen's University Belfast. The analysis of samples by ATR-FT/IR was performed at ambient temperature using a Nicolet iS5 FT-IR spectrometer Thermo Nicolet iS5 and ATR iD7 accessory (Thermo Fisher Scientific, Dublin, Ireland) , with diamond crystal, ZnSe lens, and DTGS KBr detector. After the sample holder was cleaned with an alcohol swab, the samples were placed on the flat surface of the crystal while the slip clutch tower applied equal pressure. Thirty-two scans per sample were collected in the mid-infrared range from 550 to 4000 cm^{-1} in transmission mode at a spectral resolution of 4 cm^{-1}. The collected spectra were analysed against air as background. Three replicates of each sample were measured and averaged before data pre-processing. Before the measurements of peak heights and areas, each IR spectrum was normalised, and then its second derivative was generated and auto-smoothed. The collection of the functional spectral bands associated with nutrient molecular structures, the corrections with the background spectrum and the data pre-processing were done with OMNIC 7.3 software (Spectra-Tech Inc., Madison, WI, USA).

2.4 Univariate analysis

The three spectra of each sample along with its second derivatives were read in Excel and processed by using Excel macro for peak and area measurements.[7] Peaks and areas were measured in three regions with different sub-regions and peaks (Figure 1):

(i) Carbohydrate (CHO, ca. 1482–924 cm^{-1}) region, further divided into:

- Total carbohydrate (TC, ca. 1178–941 cm^{-1}) region, containing four major peaks at ca. 1150 (TC4), 1103 (TC3), 1051 (TC2), and 1029 (TC1) cm^{-1}.

- Structural carbohydrate (STC, ca. 1482–1184 cm^{-1}), with four major peaks at ca. 1443 (STC4), 1411 (STC3), 1372 (STC2), and 1312 (STC1) cm^{-1};
- Cellulosic compounds (CEC, ca. 1294–1184 cm^{-1}) regions, with centre at ca. 1238 cm^{-1}.

Areas of each sub-carbohydrate regions (TCA, STCA, and CECA) were measured according to their respective baselines.

(ii) Protein spectra comprised the primary and secondary protein structures. The primary protein structures included amide I (AmIA) and amide II (AmIIA) with separation by ca. 1567 cm^{-1}. The baseline of protein spectral was centred at ca. 1704–1482 cm^{-1} and was used to determine peak heights in the amide region. The heights for the secondary protein structures were determined by using the second derivative function in OMNIC, according to published methods.[12,19] Amide I, amide II, α-helix (αH) and β-sheet (βS) peaks centered at ca. 1630, 1544, 1655 and 1633 cm^{-1}, respectively. The αH/βS spectral intensities and the ratios of AmI/AmII, AmIA/AmIIA were calculated.

(iii) Lipid-related region comprises two parts: carbonyl C=O region (CCO, ca. 1770–1704 cm^{-1}) and (a)symmetric CH2 and CH3 region (ASCC, ca. 2995–2760 cm^{-1}). The CCO center at ca. 1735 cm^{-1}, while ASCC features contains four major peaks centered at ca. 2959 cm^{-1} (asymmetric CH3, AsCH3), 2916 cm^{-1} (asymmetric CH2, AsCH2), 2873 cm^{-1} (symmetric CH3, SyCH3), and 2848 cm^{-1} (symmetric CH2, SyCH2). Areas of carbonyl C=O region (CCOA) and (a)symmetric CH2/CH3 region (ASCCA) were also measured according to their baselines.

2.5 Multivariate analysis

Agglomerative hierarchical cluster analysis (AHCA) and principal component analysis (PCA) were performed on each region of IR spectra to compare and distinguish inherent structural differences between both sections (stems/leaves) and cultivars. For AHCA, Ward's algorithm method was applied without any prior parametrisation of the spectral data by using squared Euclidean distance. The results of the AHCA were displayed as dendrograms and PCA results were plotted based on the two highest factor scores (PC1/PC2) and presented as a function of those scores. 95% confidence ellipses were calculated and displayed for each group (leaves/stems; cultivars) in the PCA plots. The most representative plots for each IR region were displayed. Both AHCA and PCA were carried out using PAST 4.01 software (Paleontological statistics software package, University of Oslo, Norway).[20]

2.6 Statistical analysis

For the univariate molecular analysis, Analysis of Variance (ANOVA) was performed using the statistical package of SPSS 25 (IBM Software Group, Chicago, IL, USA). Significance was declared at $P<0.05$,

and trends were declared at P≤0.10. When significant, means with different letters among cultivars were obtained using Duncan's test. The ATR-FT/IR spectroscopic data were analysed using a completely randomised design model (CRD): $Y_{ij} = \mu + T_i + e_{ij}$; where Yij was an observation of the dependent variable ij (peaks and regions), μ was the population mean for the variable; Ti was the effect of cultivar/section, as a fixed effect, and e_{ij} was the random error associated with the observation $_{ij}$. The correlation between the molecular structure profiles and the wet chemical analysis of the willow leaves and stems samples were analysed using Pearson's correlation (Appenix Table A.1). Normality test of residual data was conducted using the Shapiro−Wilk method.

3. RESULTS AND DISCUSSION

3.1 Nutritional composition

Table 1 shows the averaged nutritional composition of the four willow cultivars analysed and the stems and leaves. Overall, willow leaves showed higher (P<0.05) N and CT, and lower (P<0.05) content of fibrous compounds, including NDF, ADF, ADL, hemicellulose and cellulose. This data goes along with previous studies showing a better nutritional profile of tree's leaves in comparison with stems as animal fodder (Buxton, 1996; Johnson, 2002). No statistical differences were found between willow cultivars in the structural carbohydrate content. However, compared to the other cultivars, *S. Terra Nova* showed higher (P<0.05) N content in the stems and lower (P<0.05) content of CT in the leaves, which showed similar content than stems, resulting in a significant interaction between factors.

3.2 Carbohydrate Structure-Related Spectral Profile

Table 2 shows the carbohydrate spectral parameters of the leaves and stems of the four willow cultivars and the average values of both plant parts. Stems showed significant higher values for the three areas considered within the CHO: TCA, CECA and STCA. Likewise, most peaks in these regions showed higher (P<0.05) intensities in the stems than in the leaves. STC4 was the only exception inwhich was observed a generalised higher (P<0.001) intensity in leaves than in stems. Within cultivars, Terra Nova was the most unalike both in terms of leaves and stems, showing Beagle, Resolution and Olof samples little differences among them. Overall, Terra Nova leaves and stems exhibited higher (P<0.01) intensities in most peaks and areas of the three regions (TC, CEC, STC). However, Terra Nova leaves showed lower (P<0.05) intensities in TC1, TC4, STC1 and STC4. The interaction found between both factors in TC2, TC4, CECA and STC1 reflected differences in the intensities of some structural parameters between cultivars when stems or leaves are compared. For instance, in contrast with leaves, no differences between cultivars were found in stems of TC4 and STC1.

According to previous studies, CEC and CECA are related to the cellulosic compound of the plant,[7] whereas STC represents the NDF and ADF fractions of the vegetal material.[13] Stems of most forages showed a higher concentration of structural carbohydrates and cellulosic compounds than leaves, which generally implies lower digestibility.[21,22] The values found in univariate analysis of ATR-FTIR spectra agree with the chemical composition, as the content of NDF and ADF in the wet analysis were higher in stems samples (41.2 and 29.9% DM) than in leaves (23.1 and 13.6% DM). The cellulose content (calculated as the difference between ADF and ADL) showed a moderate correlation ($P<0.001$) with CEC height (+0.638) and area (+0.721) (Table A.2). However, no differences ($P>0.05$) were found in the fibre content between the willow cultivars either for stems or leaves. In other studies, on alfalfa, the lack of correspondence between chemical and molecular analysis of fibre content of vegetal material was attributed to changes in the strength and polarity of vibrating bonds associated with STC influenced by genetic selection transformation.[7] Nevertheless, willow, in contrast with alfalfa, is a tanniniferous forage, and the aerial parts are particularly rich in tannins.[23]

The analysis of CT by ATR-FTIR has been previously applied to different vegetal matrixes.[24,25] It is considered that one of the most representative regions for tannin quantification falls between 1577 and 1060 cm^{-1}, thus fitting with most of the CHO region, also composed of OH and C=O bonds.[26] Our results showed a positive correlation (+0.785; $P<0.001$) between the CT content and the STC4 (ca. 1443 cm^{-1}) and a negative correlation with most of the molecular structures detected. The region 1485-1425 cm^{-1} is linked to aromatic ring stretching vibrations in tannins.[27] These wavelengths, and more specifically, one prominent peak around 1445 cm^{-1}, have been highlighted as a determinant for tannin quantification by using ATR-FTIR.[26] Overall, the CT content of leaves in willow is higher than in stems,[28] and STC4 was indeed the only peak in the STC region in which leaves values showed a higher intensity. With regards cultivars, STC4 followed a similar pattern than CT content, showing lower ($P<0.05$) values for Terra Nova leaves, whereas no differences were found in the stems.

Low concentrations of CT can reduce the protein degradation in the rumen and increase the flow of feed protein and essential amino acids to the intestine;[29] however, high levels may reduce digestibility and availability of protein, palatability and intake.[4] The apparent lower content of CT in Terra Nova may thus be more beneficial for ruminant nutrition, although the effects also depend on their reactivity.[10] According to our results, CEC height and area seems to be the most suitable region for the early detection of differences in the cell wall components of the willow. Despite that, the presence of CT in the vegetal material might interfere with the proper assessment of other structures in the CHO, thus preventing for relevant correspondence with chemical analysis. At the same time, results suggest

that like in other plant materials, ATR-FTIR might be a suitable tool for predicting the CT content of tree fodders.

Figure 2 shows plots of PCA and AHCA multivariate analyses of the three carbohydrate sub-regions (TC, CEC, and STC). Plots are divided into leaves and stems to better assess differences between cultivars better. PCA allows reducing the original variable frequencies set to new ones describing trends (principal components). The first two PC of TC, CEC and STC represented in the plots explained 90.3%, 95.1% and 97.5% of population variances, respectively. Within the TC region, although some differences were observed, a complete separation between cultivars was not obtained when 95% confidence ellipses were drawn. In contrast, Terra Nova was clearly separated from the other cultivars from plots of CEC and STC region. Beagle, Resolution and Olof were similarly distributed in all regions of both sections considered, indicating no molecular structural differences in the CHO regions. Similar results were observed in the AHCA dendrograms. The distances at which Terra Nova leaves and stems samples were separated from other cultivars were higher in CEC and STC regions. However, stems of Resolution showed poor separation with Terra Nova, which was also observed in PCA plots. Indeed, although some differences ($P<0.05$) were observed in the univariate molecular structure (Table 2), Resolution showed values closer to Terra Nova in these regions than Beagle and Olof.

3.2 Protein Structure Related Spectral Profiles

Table 3 shows the protein spectral parameters of the leaves and stems of willow cultivars and the average values of both sections. Protein structure bonds contain C=O, C-N and N-H, making it unique for IR spectra analysis. AmI band absorbs around 1650 cm^{-1} and represents primarily C=O and C-N stretching vibration. AmII absorbs at ca. 1550 cm^{-1}, consisting primarily of N-H bending vibration coupled with C-N stretching vibrations.[19] The univariate analysis showed higher ($P<0.001$) value of leaves in comparison with stems in the Amide I and Amide II heights and areas, AmI/AmII ratio, and in the total Amide area. With regards cultivars, Terra Nova leaves and stems showed higher ($P<0.05$) intensities for both AmI and AmII heights and areas, and total Amide Area. When AmI/AmII ratios were calculated, only the AmI/AmII Height ratio was higher ($P<0.05$) in the stems of Terra Nova than in Beagle, and Olof.

Chemical analysis (Table 1) showed higher N content in leaves (5.06% DM) in comparison with stems (3.27% DM). Terra Nova stems also showed higher ($P<0.05$) N content in comparison with the other cultivars, whereas the higher N content of Terra Nova leaves showed only a trend ($P<0.10$). Previous studies on different forages showed lower CP content of stems than leaves, which agree with the spectral values found in the present study.[30,31] The correspondence between chemical and spectral

analyses was reflected in a moderately positive correlation (>0.70; P<0.05) between N content and Amide heights and areas, with a maximum correlation coefficient of 0.763 between N and Total amide area. In contrast, AmI/AmII height and area ratios showed a negative correlation to N content (Table A.1). Previous studies already showed a positive correlation between the N content and the Amide heights and areas in cereals.[11,32,33] However, negative[7] or no correlations[34] have also been found between amide spectral profiles and N when different alfalfa genotypes were compared. Therefore, apart from the content of N in the vegetal material, the height or area value of the amide spectral bands may be dependent on other factors, such as sources, types and processing methods of samples.[7] This fact highlights the relevance of assessing relationships for each feedstuff individually.

The secondary structure of proteins can be predicted from the AmI band vibrational frequency due to its high sensitivity to protein secondary structure.[19] The most commonly occurring protein secondary structures consist of mainly α-helices (αH) and β-sheets (βS).[12] Within AmI, the protein αH structure typically is in the range of approximately 1648–1660 cm^{-1}. For βS, the peak is in the range of approximately 1620–1640 cm^{-1}. In the current study, αH and βS peaks were detected at 1655 and 1633 cm^{-1}, respectively. The univariate analysis showed higher (P<0.001) content of both secondary structures' peaks and the αH/βS ratio in leaves than in stems. Among cultivars, both Terra Nova leaves and stems showed higher content (P<0.001) of all the protein secondary structures' parameters than the rest of cultivars . The N content was positively correlated in this case with the αH (0.741), βS (0.727) and αH/βS ratio (0.726). In forages, stems seem to have lower CP ruminal degradability than leaves, which is mainly associated with a higher content of the non-degradable protein fraction.[30,31] This study is the first exploring the protein molecular structure in willow cultivars and, therefore, no comparison can be made with previous references, as there is no information in the literature. Nevertheless, differences found in the protein secondary structures between stems and leaves were similar to that observed between cultivars' foliage, which might be related to a different CP ruminal degradability of the cultivars' foliage. Indeed, differences in protein αH and βS structures have been previously related to access to gastrointestinal digestive enzymes, which may result in different protein value and protein availability.[11,12,35] Therefore, spectral differences found by ATR-FTIR would suggest higher digestibility of leaves and Terra Nova over stems and the other cultivars, respectively. Further research is necessary to reveal the exact relationship between protein molecular structure profiles and degradation kinetics of the willow cultivars, as there are no published results.

Regarding the PCA plots and AHCA dendrograms for Amide region of willow leaves and stems samples, those presented in **Figure 3**. The first two PC for both stems and leaves explained >97% of the variance, and partly reflected differences observed between Amide structures in the univariate analysis. Terra

Nova leaves were grouped apart from the other cultivars in the PCA plot, even though they fell partially within the 95% confidence ellipse of Beagle leaves. When AHCA was applied, Terra Nova leaves were split between an independent cluster at a height around 3, and a mixed sub-group with Beagle samples at around 0.5. Regarding stems, Terra Nova samples were also well-separated but fell within the Resolution confidence ellipse. However, AHCA dendrogram clustered all Terra Nova stems in a group at a distance of 4 in comparison with the other cultivar samples. Among the other cultivars, Resolution stems showed slight differentiation with Beagle and Olof.

3.3 Lipid-Related Structure Spectral Profiles

Table 4 shows the lipid-related spectral parameters of the leaves and stems of the four willow cultivars and the average values of both sections. In mid-infrared spectrum, two regions related to lipid profiles can be detected: carbonyl C=O ester stretching region (CCO, ca. 1710–1781 cm^{-1}) and (a)symmetric CH2/3 stretching region (ASCC, ca. 3000–2761 cm^{-1}). Different patterns were found between leaves and stems for each region in the univariate analysis: stems showed higher ($P<0.05$) CCO and CCOA values; whereas all parameters within ASCC were higher ($P<0.05$) in the leaves. Terra Nova was again the most different cultivar for both lipid-related sections, although differences were not so evident as in the carbohydrate and protein regions, especially when stems were considered. Indeed, no differences ($P>0.05$) were found between stems in the height and area of CCO, and only ASCH3 (2959 cm^{-1}) intensity was different ($P<0.01$) from all the other cultivars.

It is generally assumed that leaves usually have higher fatty acids concentration compared with stems.[36] The plant cultivar also seems to affect both the total fatty acid content and the fatty acid profile.[37] When FTIR was applied previously to *Brassica carinata* seeds, it was found a positive correlation between the total fatty acid content and both CCO and ASCC regions areas.[38] In contrast, higher SyCH2 and AsCH2 heights and ASCC affected the nutritive value of alfalfa and related to its lower nutrient availability. The characteristics of lipid chain length, branching and unsaturation can be investigated by comparing contributions of lipid CH3 and CH2 functional group.[39] However, up to our knowledge, there is no data on fatty acids composition of willow leaves and stems, or among its cultivars. Further research is required to determine this and its relationship with FTIR lipid features.

Figure 4 shows PCA plots and AHCA dendrograms of both lipid-related sections for stems and leaves. The two first PC of both regions explained almost 100% of the variance (>97%). In the CCO region, leaves showed considerable variability according to the confidence ellipses, which prevented from any clear separation. This finding agreed with AHCA results. With regards stems, Terra Nova cultivar showed a different grouping trend than the other cultivars in PCA plot, being Beagle, Resolution and

Olof grouped in a pretty similar ellipse. However, AHCA dendrogram did not show apparent differences among cultivars. PCA plot of the ASCC region of leaves and stems showed similar patterns to those of CCO with a similar distribution of Beagle, Resolution and Olof. Terra Nova leaves showed different grouping trend, although it was not enough to show clear separation. AHCA dendrogram clustered most of the Terra Nova leaves and stems in the ASCC region in a different group, although distances were lower than separations observed in Protein and CHO regions, indicating lower differences. These findings of the multivariate analysis are in line with the results obtained for the different peaks and areas counts in the univariate analysis, reflecting lower differentiation of these regions among cultivars.

4. Conclusions

This study is the first revealing the nutrient molecular structure of leaves and stems from different cultivars of willow , the short rotation coppice species mostly used for bioenergy production. The use of ATR-FTIR allowed rapid detection of molecular differences between willow parts and cultivars analysed. Willow stems had higher intensities in the regions related to cellulosic compounds and a higher proportion of β-sheets, both generally related to lower dry matter and protein digestibility in rumen. Among cultivars, *Salix* "Terra Nova" showed higher intensity of the peaks and areas in the amide regions and higher αH/βS ratio in comparison with S. *Beagle*, *Olof* and *Resolution*, which may be related to higher protein content and digestibility. However, *Terra Nova* also showed a higher intensity of cellulosic structures. Finally, the information provided by the STC region seems to be related to the content of condensed tannins, which appears to be lower in stems and *Terra Nova* in comparison with leaves and the other cultivars, respectively. The application of ATR-FTIR to willow foliage allows the obtention of relevant chemical and molecular information in a quicker way and from a higher amount of samples, which makes possible to monitor the variability between plant parts and cultivars. The easiness to collect more information would contribute to better understand the relationship between nutritional composition and animal performance, crucial for improving the efficiency of agroforestry systems. Further *in vivo* animal studies are needed to assess the relationship between the molecular structural parameters investigated and the specific benefits of the willow as a novel forage crop for ruminants.

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Figure legends

Fig. 1. Spectra of willow cultivars' leaves (a) and stems (b) with annotations of the peaks and areas analysed.

Fig. 2. Principle component analysis (PCA) plots and agglomerative hierarchy cluster analysis (AHCA) dendrograms of carbohydrate regions of willow cultivars' leaves (Beagle (○); Terra Nova (◊); Olof (□); Resolution (Δ)). TC, total carbohydrate (ca. 1184–924 cm^{-1}); CEC, cellulosic compounds (ca. 1294–1184 cm^{-1}); STC, structural carbohydrate (baseline ca. 1482–1184 cm^{-1}); (**a**) PCA dendrogram of TC; (**b**) AHCA plot of TC; (**c**) PCA dendrogram of CEC; (**d**) AHCA plot of CEC; (**e**) PCA dendrogram of STC; (**f**) AHCA plot of STC.

Fig. 3. Principle component analysis (PCA) plots and agglomerative hierarchy cluster analysis (AHCA) dendrograms of carbohydrate regions of willow cultivars' stems (Beagle (○); Terra Nova (◊); Olof (□); Resolution (Δ)). TC, total carbohydrate (ca. 1184–924 cm^{-1}); CEC, cellulosic compounds (ca. 1294–1184 cm^{-1}); STC, structural carbohydrate (baseline ca. 1482–1184 cm^{-1}); (**a**) PCA dendrogram of TC; (**b**) AHCA plot of TC; (**c**) PCA dendrogram of CEC; (**d**) AHCA plot of CEC; (**e**) PCA dendrogram of STC; (**f**) AHCA plot of STC.

Fig. 4 Principal component analysis (PCA) plots and agglomerative hierarchical cluster analysis (AHCA) dendrograms of amide region of willow cultivars (Beagle (○); Terra Nova (◊); Olof (□); Resolution (Δ)) separated by stems and leaves. Baseline of amide region is ca. 1704-1482 cm^{-1}; (**a**) PCA dendrogram of the leaves; (**b**) AHCA plot of the leaves; (**c**) PCA dendrogram of the stems; (**d**) AHCA plot of the stems.

Fig. 5 Principle component analysis (PCA) plots and agglomerative hierarchy cluster analysis (AHCA) dendrograms for the CCO, carbonyl C=O region (ca. 1710–1781 cm^{-1}) of willow cultivars (Beagle (○); Terra Nova (◊); Olof (□); Resolution (Δ)) separated by stems and leaves. (**a**) PCA dendrogram of leaves; (**b**) AHCA plot of leaves; (**c**) PCA dendrogram of stems; (**d**) AHCA plot of stems.

Fig. 6 Principle component analysis (PCA) plots and agglomerative hierarchy cluster analysis (AHCA) dendrograms for the ASCC (asymmetric and symmetric CH2 and CH3) region (ca 3000–2761 cm^{-1}) of willow cultivars (Beagle (○); Terra Nova (◊); Olof (□); Resolution (Δ)) separated by stems and leaves.;. (**a**) PCA dendrogram of leaves; (**b**) AHCA plot of leaves; (**c**) PCA dendrogram of stems; (**d**) AHCA plot of stems.

Table 1. Chemical composition (g/100 g DM unless specified) of leaves and stems of willow cultivars.

	PLANT PART	CULTIVAR				s.e.m.	Pcult	Ppart	Pcxp
		BEAGLE	TERRA NOVA	OLOF	RESOLUTION				
DM	LEAVES	15.3	15.2	14.7	14.6	0.658	N.S.	N.S.	N.S.
g/100 g	STEMS	17.9	17.8	18.3	18.2				
N	LEAVES	4.59x	5.82x	4.73x	4.76x	0.192	*	***	*
	STEMS	2.61by	4.36ay	3.15by	3.25by				†
ASH	LEAVES	0.97y	1.02y	1.02y	1.16y	0.041	N.S.	***	N.S.
	STEMS	1.17x	1.14x	1.30x	1.30x				
CT	LEAVES	12.4cx	6.71a	10.8bcx	12.0bx	0.909	**	***	**
	STEMS	4.83y	5.22	5.04y	5.81y				
NDF	LEAVES	24.2y	24.2y	21.3y	22.9y	4.467	N.S.	***	N.S.
	STEMS	39.2x	42.1x	38.8x	44.7x				
ADF	LEAVES	14.1y	14.4y	11.9y	14.2y	3.507	N.S.	***	N.S.
	STEMS	28.0x	30.1x	28.3x	33.3x				
ADL	LEAVES	7.93y	7.83	5.19	6.89	3.891	N.S.	**	N.S.
	STEMS	13.9x	17.0	14.7	16.7				
HEMIC	LEAVES	10.1y	9.76y	9.38y	8.69y	1.319	N.S.	*	N.S.
	STEMS	11.3x	12.0x	10.5x	11.5x				
CEL	LEAVES	6.21y	6.56y	6.71y	7.28y	0.904	N.S.	***	N.S.
	STEMS	14.1x	13.1x	13.6x	16.6x				

s.e.m., standard error of mean; Pcult: p-value of cultivar; Ppart: p-value of plant part

a,b,c Values with same letter in each row mean not significantly different at P> 0.05; x,y Values within same letter in each column mean not significantly different at P> 0.05

DM = dry matter; N = nitrogen; CT = condensed tannins; NDF = neutral detergent fibre; ADF = acid detergent fibre; ADL = acid detergent lignin; HEMIC = hemicellulose (calculated as NDF-ADF); CEL = cellulose (calculated as ADF-ADL).

503 **Table 2**. Carbohydrate structural parameters of leaves and stems of willow cultivars.

		PLANT PART	CULTIVAR				s.e.m.	Pcult	Ppart	Pcxp
			BEAGLE	TERRA NOVA	OLOF	RESOLUTION				
TC REGION[1]	**TC1**	LEAVES	0.635ay	0.606by	0.635ax	0.637ax	0.0033	***	***	N.S.
		STEMS	0.688x	0.674x	0.688y	0.682y				
	TC2	LEAVES	0.469by	0.525a	0.474by	0.481b	0.0051	***	**	*
		STEMS	0.490bx	0.540a	0.496bx	0.463c				.
	TC3	LEAVES	0.320by	0.351ay	0.324by	0.327by	0.0042	***	***	N.S.
		STEMS	0.354bx	0.403ax	0.354bx	0.351bx				
	TC4	LEAVES	0.127ab	0.112cy	0.130a	0.122by	0.0020	**	***	***
		STEMS	0.132	0.133x	0.127	0.131x				
	TCA	LEAVES	73.76y	75.39y	74.68y	73.54y	0.3794	***	***	N.S.
		STEMS	81.04bx	83.70ax	81.62bx	80.75bx				
CEC REGION[2]	**CEC**	LEAVES	0.076by	0.088ay	0.075by	0.076by	0.0021	***	***	N.S.
		STEMS	0.095bx	0.114ax	0.098bx	0.101bx				
	CECA	LEAVES	3.823y	4.048y	3.655y	3.831y	0.0848	***	***	*
		STEMS	4.943cx	5.896ax	5.186bcx	5.416bx				
STC REGION[3]	**STC1**	LEAVES	0.102ay	0.090by	0.102ay	0.109ay	0.0017	*	***	***
		STEMS	0.125x	0.135x	0.127x	0.131x				
	STC2	LEAVES	0.137bcy	0.149ay	0.134cy	0.142by	0.0022	***	***	N.S.
		STEMS	0.151bx	0.160ax	0.148bx	0.153bx				
	STC3	LEAVES	0.128b	0.160a	0.125b	0.129b	0.0029	***	***	N.S.
		STEMS	0.138b	0.171a	0.133b	0.140b				

STC4	LEAVES	0.160ax	0.149bx	0.157ax	0.167ax	0.002	**	***	N.S.
	STEMS	0.125y	0.117y	0.122y	0.123y				
STCA	LEAVES	29.97ab	31.41ay	29.30by	31.21a	0.3683	***	***	N.S.
	STEMS	31.25b	33.99ax	30.91bx	32.14b				

s.e.m., standard error of mean; Pcult: p-value of cultivar; Ppart: p-value of plant part

a,b,c Values with same letter in each row mean not significantly different at P> 0.05; x,y Values within same letter in each column mean not significantly different at P> 0.05

[1] Four major peaks at ca. 1029 (TC1) 1051 (TC2), 1103 (TC3) and 1150 (TC4) cm^{-1} in TC region. TCA, peak area of TC region.

[2] CEC, cellulosic compounds (ca. 1238 cm^{-1}); CECA, peak area of CEC region.

[3] Four major peaks at ca. 1312 (STC1), 1372 (STC2), 1411 (STC3) and 1443 (STC4) cm^{-1}. STCA, peak area of STC region.

Table 3. Protein region structural parameters of leaves and stems of willow cultivars.

	PLANT PART	CULTIVAR				s.e.m.	Pcult	Ppart	Pcxp
		BEAGLE	TERRA NOVA	OLOF	RESOLUTION				
Amide I Height	LEAVES	0.285bx	0.322ax	0.287bx	0.275bx	0.0059	***	***	N.S.
	STEMS	0.159by	0.232ay	0.105by	0.101by				
Amide II Height	LEAVES	0.208bx	0.247ax	0.208bx	0.206bx	0.0062	***	***	N.S.
	STEMS	0.106by	0.132ay	0.105by	0.101by				
AmI/AmII Height	LEAVES	1.379ay	1.303by	1.385a	1.348aby	0.0398	N.S.	***	**
	STEMS	1.556bx	1.793ax	1.522b	1.738abx				
Amide I Area	LEAVES	30.44bx	34.06ax	30.54bx	30.44bx	0.5331	**	***	*
	STEMS	20.49by	28.24ay	20.37by	21.76by				
Amide II Area	LEAVES	12.30bx	14.34ax	12.37bx	12.24bx	0.2898	***	***	N.S.
	STEMS	6.612by	8.858ay	6.327by	6.588by				
AmI/AmII Area	LEAVES	2.496ay	2.380by	2.481aby	2.503ay	0.0330	N.S.	***	N.S.
	STEMS	3.158x	3.208x	3.223x	3.315x				
Amide Area	LEAVES	42.74bx	48.40ax	42.91bx	42.68bx	0.8131	***	***	N.S.
	STEMS	27.10by	37.09ay	26.69by	28.35by				
α-Helix	LEAVES	0.283bx	0.322ax	0.287bx	0.271bx	0.0064	*	***	N.S.

	STEMS	0.148by	0.211ay	0.147by	0.154by				
β-Sheet	LEAVES	0.330bx	0.360ax	0.334bx	0.322bx	0.0059	***	***	*
	STEMS	0.195by	0.269ay	0.193by	0.202by				
αH/βS	LEAVES	0.855bx	0.896ax	0.861bx	0.842bx	0.0061	***	***	N.S.
	STEMS	0.754by	0.783ay	0.762aby	0.761by				

511 s.e.m., standard error of mean; Pcult: p-value of cultivar; Ppart: p-value of plant part

512 a,b,c Values with same letter in each row mean not significantly different at P> 0.05; x,y Values within same letter in each
513 column mean not significantly different at P> 0.05

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Table 4. Structural parameters of lipid-related regions of leaves and stems of willow cultivars.

		PLANT PART	CULTIVAR				s.e.m.	Pcult	Ppart	Pcxp
			BEAGLE	TERRA NOVA	OLOF	RESOLUTION				
CCO REGION[1]	**CCO**	LEAVES	0.054b	0.068a	0.053b	0.050by	0.0029	**	*	N.S.
		STEMS	0.062	0.065	0.061	0.061x				
	CCOA	LEAVES	1.219by	1.659ay	1.187by	1.169by	0.1071	*	***	N.S.
		STEMS	2.017x	2.052x	2.012x	2.015x				
ASSCA REGION[2]	**SyCH2**	LEAVES	0.098bx	0.129ax	0.093bx	0.091bx	0.0052	***	***	*
		STEMS	0.052aby	0.057ay	0.044by	0.046by				
	SyCH3	LEAVES	0.061bx	0.069ax	0.057bx	0.058bx	0.0008	***	***	*
		STEMS	0.049aby	0.051ay	0.046by	0.046by				
	AsCH2	LEAVES	0.149bx	0.186ax	0.152bx	0.139bx	0.0074	***	***	N.S.
		STEMS	0.079aby	0.087ay	0.067by	0.070by				
	AsCH3	LEAVES	0.045bcx	0.049ax	0.047abx	0.041cx	0.0011	***	***	N.S.
		STEMS	0.026by	0.030ay	0.025by	0.024by				
	ASCCA	LEAVES	11.20bx	13.17ax	10.64bx	10.42bx	0.3673	***	***	N.S.
		STEMS	7.290aby	7.833ay	6.592by	6.695by				

s.e.m., standard error of mean; Pcult: p-value of cultivar; Ppart: p-value of plant part

a,b,c Values with same letter in each row mean not significantly different at P> 0.05; x,y Values within same letter in each column mean not significantly different at P> 0.05

[1] CCO, carbonyl C=O (centres at ca. 1735 cm^{-1}); CCOA, peak area of CCO region (baseline ca. 1770–1704 cm^{-1})

[2] SyCH2, symmetric CH2 (ca. 2848 cm^{-1}); SyCH3, symmetric CH3 (ca. 2873 cm^{-1}); AsCH2, asymmetric CH2 (ca. 2916 cm^{-1}); AsCH3, asymmetric CH3 (ca. 2959 cm^{-1}); ASCCA, peak area of asymmetric and symmetric CH2 and CH3 (baseline ca. 2995–2760 cm^{-1})

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