

Prediction of starch reserves in intact and ground grapevine cane wood tissues using near infrared reflectance spectroscopy (NIRS)

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Running title: Development of a prediction tool for starch reserves in grapevine

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Abstract

BACKGROUND: Near Infrared Reflectance Spectroscopy (NIRS) technology can be a powerful analytical technique for the assessment of plant starch, but generally samples need to be freeze-dried and ground. This study investigated the feasibility of using NIRS technology to quantify starch concentration in ground and intact grapevine cane wood samples (with or without the bark layer). A partial least squares (PLS) regression was used on the sample spectral data and was compared against starch analysis using a conventional wet chemistry method.

RESULTS: Accurate calibration models were obtained for the ground cane wood samples ($n = 220$), one based on 17 factors ($R^2 = 0.88$, root mean square error of validation (RMSEV) of 0.73 mg.g^{-1}) and the other based on 10 factors ($R^2 = 0.85$, RMSEV of 0.80 mg.g^{-1}). In contrast, the prediction of starch within intact cane wood samples was very low ($R^2 = 0.19$). Removal of the cane bark tissues did not substantially improve the accuracy of the model ($R^2 = 0.34$). Despite these poor correlations and low ratio of prediction to deviation (RPD) values of 1.08-1.24, the root mean square error of cross-validation (RMSECV) values were 0.75-0.86 mg.g^{-1}) indicating good predictability of the model.

CONCLUSION: As indicated by low RMSECV values, NIRS technology has the potential to monitor grapevine starch reserved in intact cane wood samples.

Keywords: carbohydrates, cane wood, Pinot Noir, Chardonnay,

Introduction

Deciduous woody species (such as *Vitis vinifera*) rely exclusively on starch reserves accumulated in summer to support new root and shoot growth in the following spring.¹⁻⁴ Grapevine starch reserves stored in cane wood and root have been shown to vary within and between seasons depending on climatic and management factors⁵⁻⁶ and also between varieties.² Therefore, knowledge of starch level in winter would provide opportunities to make informed management decisions for the subsequent season.

Traditionally, quantification of starch concentration has relied on wet chemistry analytical methods based on colourimetric or chromatography analyses.^{4,7-8} These methods are time consuming and labour intensive with samples typically dried and ground before extraction with a solvent in the laboratory. Recent work has highlighted the lack of consistency in starch measurement among wet chemistry methods and laboratories.⁹⁻¹⁰ Alternative approaches have been explored including reflectance spectroscopy combined with on-solid iodine complexation¹¹ and *in vivo* x-ray micro CT technology¹². Chemometrics combined with near-infrared reflectance spectroscopy (NIRS) has the potential to provide a faster and more reliable approach for quantification of starch concentration in various tissues.¹³⁻¹⁴

NIRS is a powerful technique that has enabled rapid analysis of various compositional parameters in wine grapes, must and grapevine tissues^{13, 15-16}, grapevine water potential¹⁷⁻¹⁹ and starch. Accurate predictive models of starch have been reported for a range of woody tree systems including grapevine, *Vitis* spp.^{8, 20-21}, 73 tree species²² and *Eucalyptus globulus*²³ using dried and ground samples. It is currently unknown if NIS can be used to predict starch on intact cane wood samples though this technology has been used to successfully predict a range of chemical and physical wood properties of intact timber samples. The accuracy of the models was influenced by factors such as sample surface preparation, sample thickness and surface texture.²⁴⁻²⁶

In contrast to conventional wet chemistry methods, NIRS could allow analysis of a large number of samples as sample preparation is relatively straightforward and no chemical reagents are necessary. Further, the possibility of simplifying sample presentation i.e. using whole canes instead of ground samples could dramatically increase sample throughput and potentially allow for non-destructive *in vivo* measurement in the field with a portable NIRS.

In experiments described here, starch concentration of ground and intact grapevine cane wood tissues was analysed using a traditional wet chemistry method as well as by spectral analysis to explore the potential for using multivariate data analysis to develop a rapid tool for the estimation of starch concentration in intact and ground grapevine tissues. To our knowledge, this is the first study to report the feasibility of NIR spectroscopy for the

determination of starch in intact cane wood samples, with or without bark. We hypothesised that removing bark, which could interfere with the NIR starch signal, may improve the accuracy of the predictive model.

Materials and methods

Plant material

In 2010 (n = 59), 2011 (n = 107) and 2012 (n = 39), cane wood samples were sourced from five commercial vineyards in southern Tasmania, Australia (vineyard a: 42°37'55"S, 146°48'28"E; vineyard b: 42°48'28"S, 147°25'42"E; vineyard c: 42°45'49"S, 147°23'15"E; vineyard d: 42°48'28"S, 147°25'26"E; vineyard e: 42°49'11"S, 147°50'32"E). These samples were chosen to represent a range of analytical values, growing years, conditions (aspect, soil, vine age (i.e. 8 to 16 years-old) and vigour), pruning systems (cane and spur pruning) and grape varieties (i.e. Pinot Noir and Chardonnay growing on own roots). The climate is cool temperate with an annual mean rainfall of ~600 mm (Australian Bureau of Meteorology, www.bom.gov.au). The rainfall in 2010, 2011, 2012 and 2018 was 531, 759, 595.5 and 535.2 mm, respectively (www.bom.gov.au). It is worthy to note that the total annual rainfall in 2011 was 27% higher than the long-term average value. Mean daily maximum/minimum temperatures are 22.5/12.5 °C and 12.0/4.0 °C in summer and winter, respectively (www.bom.gov.au). To minimize tissue variability due to unstable phenological phases, all wood samples were collected during winter dormancy at pruning (June).

Additional samples harvested in 2018 (n = 148) were sourced from vineyard c, Pinot Noir block. The vines were 18-years-old and cane pruned.

Sample preparation for grinding

Cane internodes (excluding buds) collected in 2010-2012 were cut into approximately 3 cm segments, and freeze-dried using a freeze-drier (Christ beta 1-8LD plus). These samples were firstly ground to be able to pass through a 1 mm sieve using an IKA Cutting Mill (A11 basic Analytical mill) then all samples were finely ground into a powder using a Mixer Mill MM 200 (Retsch, Haan, Germany). Samples from 2018 were freeze-dried as above, then directly ground into a fine powder with a Mixer Mill MM 200. All ground samples were stored at -20 °C pending NIR analysis, which was carried out within one month following collection in each season. Every tenth sample was done in triplicate to assure quality control of the laboratory method. The laboratory error of 0.19 mg.g⁻¹ was determined by the average standard deviation of the triplicate analysis.

Spectral measurements

NIRS analysis was performed using a FT-NIR spectrophotometer (Bruker MPA, Ettlingen, Germany) at ambient temperature (~20 °C). Ground samples were placed into 7 mL glass vials for NIRS analysis. For the fresh intact cane wood samples, NIR spectra of each sample were recorded using a fibre optic probe at five different positions along the cane segment, which were then averaged to a single spectrum. Immediately after, this procedure was

repeated on the same sample after the bark had been removed using a scalpel. Absorbance spectra were taken from 12,500 to 4000 cm^{-1} with a spectral resolution of 8 cm^{-1} and 64 scans (vials) or 5 x 4 scans (fibre optic probe) per sample. The intact samples were then progressed to freeze drying and grinding as explained previously.

Chemical analysis

Soluble sugars were extracted from 100 mg powdered samples in 3 mL of 80% ethanol incubated in a water bath (60 °C) for 1 hour and then centrifuged (4000 g) for 10 min at 16 °C. The extraction was repeated twice. The concentration of insoluble starch (expressed in mg g^{-1} of glucose equivalents) was analysed using a commercial enzyme assay kit (Total Starch Assay Procedure, K-TSTA-50A/K-TSTA-100A 08/16, Megazyme International, Bray, Ireland). The samples were determined with a Genesys 10S UV-Vis spectrometer (Thermo Fisher Scientific, Madison, WI, USA). Standardized regular maize starch control was used for the calibration curve. A D-(+)-glucose standard (1.0 mg/mL in 0.2% (w/v) benzoic acid) was run with every batch of 10 samples. The starch concentrations are reported as the percentage of dry matter.

Chemometrics and data analysis

A partial least square (PLS) regression model was created correlating the quantitative starch data with the corresponding NIR spectra using the Unscambler® X software (version 10.1, CAMO Software, Oslo, Norway). Principal component analysis was performed to detect

potential spectral outliers²⁷, defined as those data points having a Mahalanobis distance (i.e. data point to centroid) greater than three.²⁸

We used the NIPALS algorithm (Non-linear Iterative PLS) for the determination of loadings and scores. The scores and loadings were calculated pair-by-pair by an iterative procedure. For the ground cane wood starch model, the data set was randomly divided into two groups prior to applying the PLS procedure – one group would be used for either the calibration model (n = 220) or the external validation model (n = 133) to assess the calibration equation as a prediction model. We chose the external validation approach over the cross-validation procedure to obtain true independent validation²⁹. For the intact cane wood samples, cross-validation of the model was preferred due to overall small sample number.

Numerous calibration models were trialled using different and a combination of spectral – pre-processing and spectral ranges. The performance of the validation and cross-validation models were evaluated by the root mean square error of validation (RMSEV) or the root mean square error of cross-validation (RMSECV), the bias, ratio of performance deviation (RPD), the regression coefficient (R^2) between the predicted and reference values and RMSEV relative to the laboratory error. RMSEV or RMSECV expresses the average error of validation that is indicative of the error for future predictions when the model is applied to unknown samples.³⁰ RPD (calculated from the ratio of the SD of laboratory references values to RMSEV or RMSECV) is a useful statistic that gives an assessment of the predictive power of the model relative to the data range. The RPD values should be larger than 2.5, however, lower RPD values can result from a narrow range of the reference values (small

SD) or to a large error in the prediction (RMSEV or RMSECV) as compared with the variability of the reference values³¹⁻³².

Results

Ground cane wood samples

Table 1 shows the range between maximum and minimum values, mean and SD for starch concentration of the calibration and the validation sets analysed by reference methods.

Chemical variation found in the reference analyses could be considered acceptable and wide enough for the development of the aimed calibration equations for the ground cane wood samples however, the range of the intact cane wood samples was more limited (i.e. 5.9 to 8.3%) (Table 1). Figure 1 shows the absorbance spectra of all ground cane wood samples. The wave number range of 4250–8000 cm^{-1} was used to develop the calibration models.

The score plots of Factors 1 and 3 of the starch prediction model indicate that clustering of spectral data was evident for growing season (Fig. 2) but not variety, pruning method and vineyard (data not presented). Despite this clustering, PLS regression of NIRS spectra and laboratory values produced a good calibration model (Fig. 3; Table 2). The best calibration model based on high R^2 and low RMSEV was achieved by using a Multiplicative Scatter Correction (MSC) for the spectral range between 4246-9404 cm^{-1} . A 17-factor model gave the highest R^2 and the lowest RMSEV (Model A; $R^2 = 0.88$, RMSE = 0.73 mg.g^{-1}), however, a much simpler model comprising of only 10 factors (Model B) produced an only marginal lower R^2 of 0.85 and a slightly higher RMSEV of 0.80 mg.g^{-1} (Table 2). The RPD values of

both models were above 2.5 (Table 2). The histogram plot of starch concentrations of ground cane wood samples (Appendix 1a) showed a non-normal distribution due to the skew towards high measured starch results.

Factors 1 to 3 explained 64% of the variation (i.e. Factor 1: 49% (variance), 13% (contribution to the model), Factor 2: 47%, 10%, Factor 3: 3%, 41%). Factors 1 to 3 and in particular, Factor 3, show that the major regions of interest occurred between 4000 to 8000 cm^{-1} (Fig. 4), a region that contains many vibrational signals related to starch.³³ In particular, dominant bands attributed to starch included 5300-5200 cm^{-1} (amylose and amylopectin helix structures), 4700-4600 cm^{-1} (C-H stretching and C-O stretching combination) and 4420-4283 cm^{-1} (C-H stretching and CH_2 deformation combination band). In addition, the complexity of the model suggests that it most likely also includes variation in lignin and cellulose.

Intact cane wood samples

NIR spectra of intact wood sample correlated poorly with the starch values as indicated by the low R^2 (0.19) and RPD (1.08) values (Table 1). The removal of bark did not improve the accuracy of the predictive model (0.34; Table 1). However, the low RMSEV values ($< 0.85 \text{ mg.g}^{-1}$) of intact cane wood indicate similar predictability of the model, and was comparable to the larger data set of ground cane wood samples. The histogram plot of starch concentrations for intact cane wood show a non-normal distribution with a skew towards medium values due to a lack of low starch concentrations $< 6\%$ (Appendix 1).

Discussion

A good predictive model was obtained for freeze dried and ground cane wood, as similarly reported for ground grapevine trunk tissues using PLS and NIRS ($R^2 = 0.79$ and RMSEP= 1.29% DW²¹), and PLS and ATR-FT-MIR ($R^2 = 0.95$ and RMSEP= 1.43% DW²⁰). While the predictability of Model B was better than Model A, Model A is more robust in practical applications as it is based on 10 factors only. Histogram plots of starch concentrations show a non-normal distribution due to the skew towards the high measured starch results. Therefore, the accuracy of model A could be further improved by expanding the dataset to include additional low starch values. Despite the first three factors explaining 99% of the spectral variation, a further seven were required to improve the accuracy of the model – such a high number most likely reflects the complexity of the data set, which included samples obtained across four growing seasons, two grape varieties, two pruning systems and five commercial vineyards. Although only growing season was found to cause clustering of the spectral data (most likely due to differences in total annual rainfall, particularly in 2011), the other factors are also likely to contribute to the variation of the data set. Nonetheless, the RPD values of both models were above 2.5, indicating that both models are very reliable. The relative error of RMSEV versus laboratory error of the triplicate starch measurements was surprisingly high ranging from 3.8 to 4.2, however this is partly due to a very low laboratory error (i.e. 0.19 mg.g⁻¹) as determined in this study as compared to previous studies which are much higher, ranging from e.g. 3-5 mg.g⁻¹.²³

The correlation of NIR spectra to starch concentration for intact cane wood samples was poor, even with the removal of the bark layer. A closer examination of the distribution of starch granules in cane wood confirm that they are largely occurring in the ray parenchyma of the xylem and phloem (Fig. 5). For such non-homogenous samples, capturing this large variation may be challenging, particularly as the penetration depth into the sample of reflectance measurements is quite small. According to the literature, the penetration depth of wood tissue and powders can range from 300 μm up to 4 mm depending on the physical, optical and composition characteristics of the sample.³⁴⁻³⁵

NIRS analysis of intact *Pinus taeda* wood samples showed poor calibrations of specific gravity and insoluble lignin, most likely due to variation in moisture content and surface roughness.³⁶ In contrast, good calibrations (R^2 : 0.75 - 0.84, SEC:1.02-1.37) were developed for predicting extractives, lignin and cellulose contents from solid wood samples of *E. globulus*.²⁴ Strong calibrations of physical properties (e.g. density, modulus of elasticity and modulus of rupture) were obtained for intact wood surfaces (both radial and transverse), particularly using a larger-sized probe (spot size of 10 mm) which enabled it to capture more sample variability.²⁶ In this study, the probe circular spot size was 3 mm, and we addressed the heterogeneity of the cane wood surface by analysing 5 different spots. However, this could not address the variation in the longitudinal distribution of starch in the sample.

Despite the poor correlation and low RPD, it is worthy to note that the RMSECV values of intact cane wood samples were similar to those obtained for ground cane wood samples

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suggesting good predictability of the model. As shown in Appendix 1, there was a non-normal distribution with a skew towards medium values in the intact cane wood samples. Previous work has demonstrated RPD to be a less reliable indicator of the quality of the model for non-normally distributed datasets.³⁷ As such, future work should aim to include a more even distribution of a large range of starch concentrations to improve the correlation. Further, increased sampling that includes season and variety variability would also allow the development of a more robust model, as has already been developed for ground plant samples.^{9,14,21} Therefore, it is still worth pursuing the use of intact samples for predicting starch concentration by NIRS, if not for non-destructive real time measurements in the field, but to reduce the sample preparation time of collected samples. Collecting NIR spectra from transverse and/or longitudinal sections of the cane wood rather than from the outer surface of the cane wood, as done in this study, may also help improve the correlation. Similar to the standard procedure used for intact timber core increments, these intact cane wood samples could also be dried to reduce the effect of moisture content on the accuracy of the model. While the values associated with ground wood calibrations have shown to be generally higher than those obtained for solid wood²⁴, the advantages to using solid wood calibrations may outweigh the slight decrease in accuracy.

Conclusions

Consistent with previous studies, the current results show that NIRS technique is suitable for the determination of grapevine reserve carbohydrate of ground cane wood samples, despite clustering across growing year. For the intact cane wood samples, while only a single season

was examined, the results indicated that, despite poor calibrations, NIRS has the potential to assess the starch content of intact cane stems as evidenced by low RMSECV values. Contrary to our hypothesis, removal of the cane bark tissues did not substantially improve the accuracy of the model.

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Table 1. Statistical summary for the parameters used in the present paper, separately showing the a) calibration and b) validation datasets. SD = standard deviation. Standard error of laboratory = 0.19 mg.g⁻¹

	Sample (n)	Mean	Min	Max	SD
<i>Calibration data set</i>					
Starch concentration (mg.g ⁻¹) of <i>ground</i> cane wood samples (2010-2012 & 2018)	220	6.7	1.8	10.4	1.87
<i>Validation data set</i>					
Starch concentration (mg.g ⁻¹) of <i>ground</i> cane wood samples (2010-2012 & 2018)	133	6.9	2.1	10.4	2.08
Starch concentration (mg.g ⁻¹) of <i>intact</i> cane wood samples (2018 only)	147	8.2	5.9	10.8	0.93

Table 2. Calibration statistics for the estimation of starch concentration (mg.g^{-1}) for ground and intact cane wood (*Vitis vinifera*) obtained by PLS regression.

Sample type	Bias	$R^2(\text{V})$ or $R^2(\text{C})$	RMSEV or RESEC (mg.g^{-1})	RPD
Ground cane wood samples*				
Independent validation Model A (10 factors)	-0.01	0.85	0.80	2.6
Independent validation Model B (17 factors)	-0.05	0.88	0.73	2.85
2018 intact cane wood (with bark)				
Cross-validation data	0.004	0.20	0.86	1.08
2018 intact cane wood (no bark)				

Cross-validation data	0.001	0.36	0.75	1.24
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* included data from 2010, 2011, 2012 and 2018 growing seasons, RPD (residual predictive deviation) = SD of laboratory values/RMSEV or RMSEC

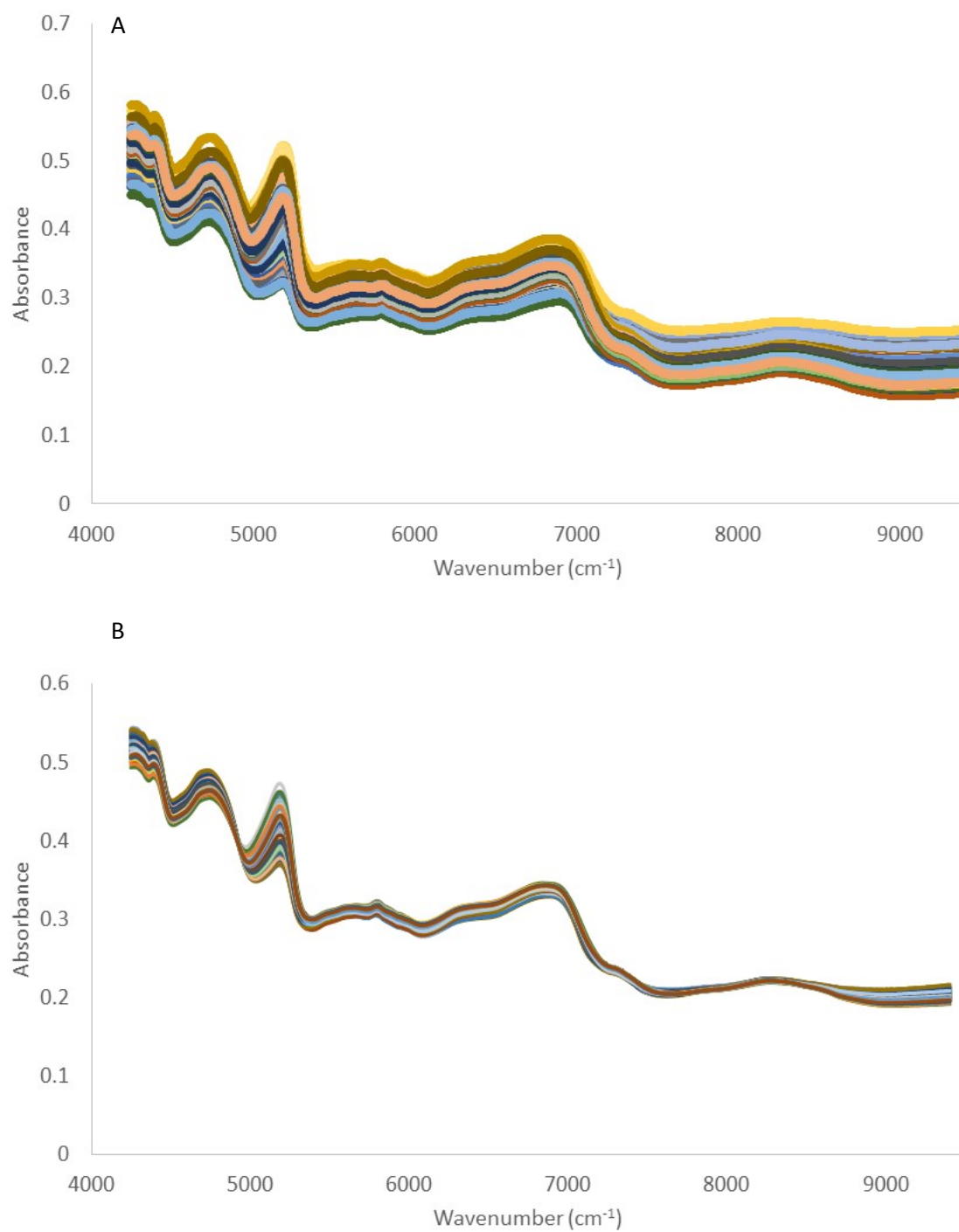


Figure 1. Absorbance (A) raw, and (B) multiplicative scatter correction spectra acquired for ground cane wood (*Vitis vinifera*) collected over four seasons (2010, 2011, 2012 and 2018).

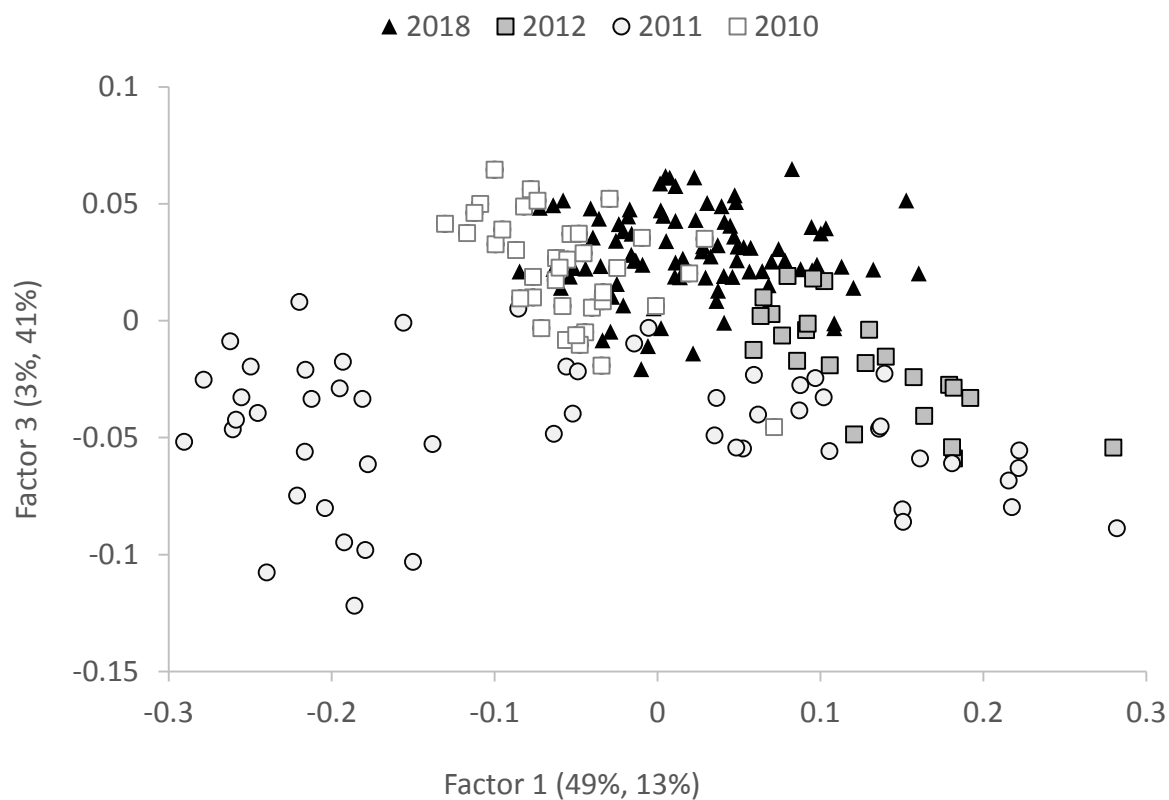
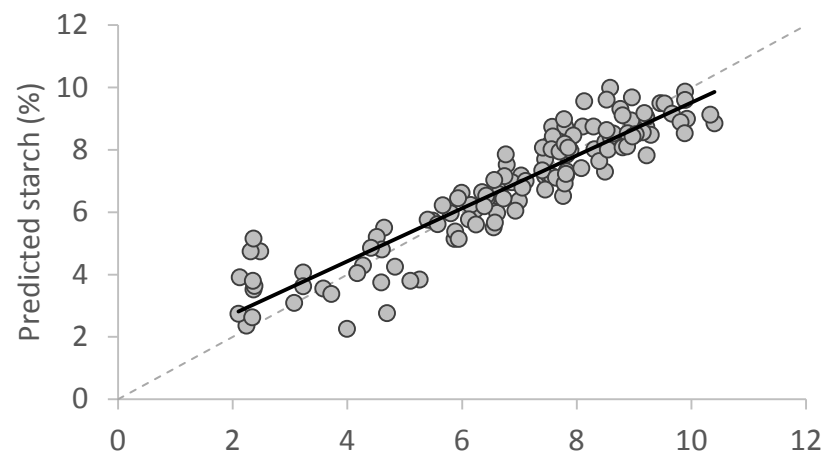
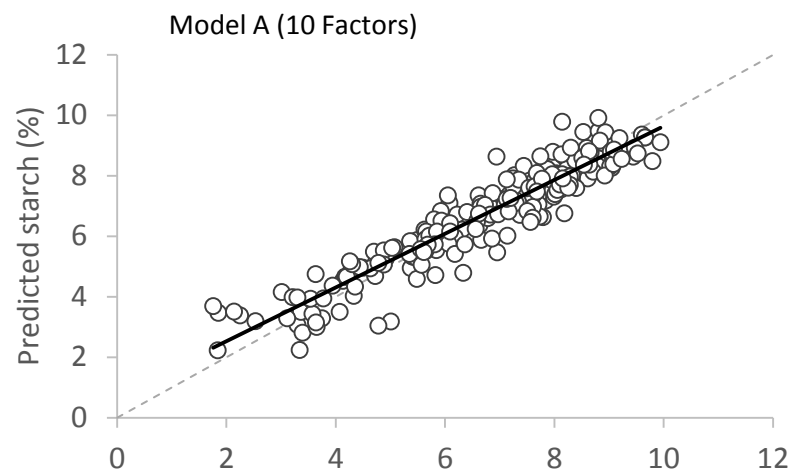


Figure 2. PLS score plot for starch concentration (%) of ground cane wood (*Vitis vinifera*) obtained across four growing seasons [2010 (open square), 2011 (circle), 2012 (grey square) and 2018 (black triangle)].



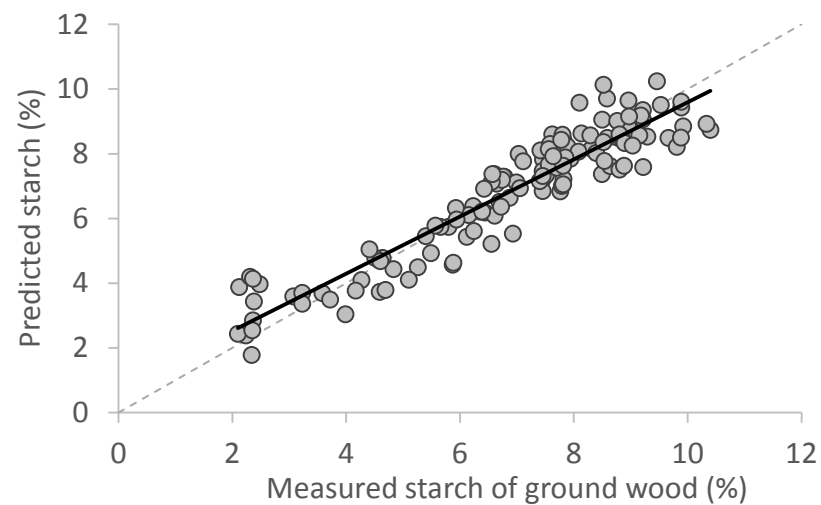
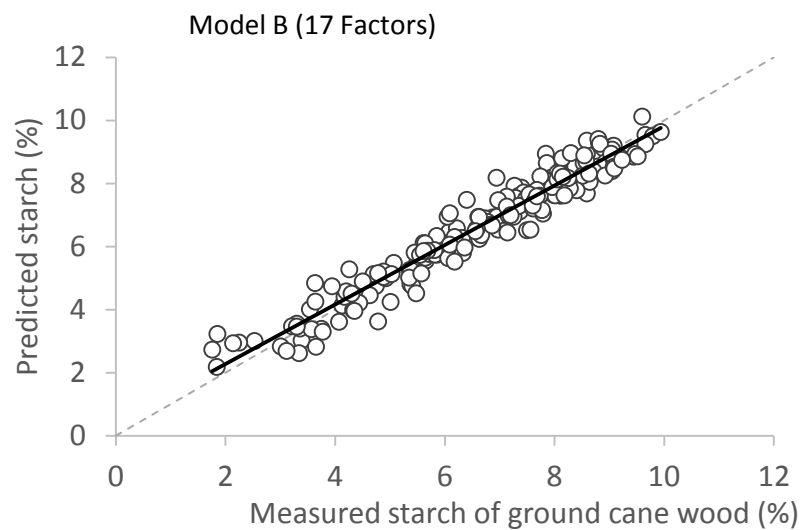


Figure 3. Relationship between starch concentration (%) of ground cane wood (*Vitis vinifera*) measured with the standard reference methods (x-axis) and those predicted by NIR (y-axis) for data collected over four seasons (2010, 2011, 2012 and 2018) for Model A (10 Factors) and Model B (17 Factors). Linear fit in black while dotted line fit 1:1 line. N = 220 and 133 for predicted and independent validation models, respectively.

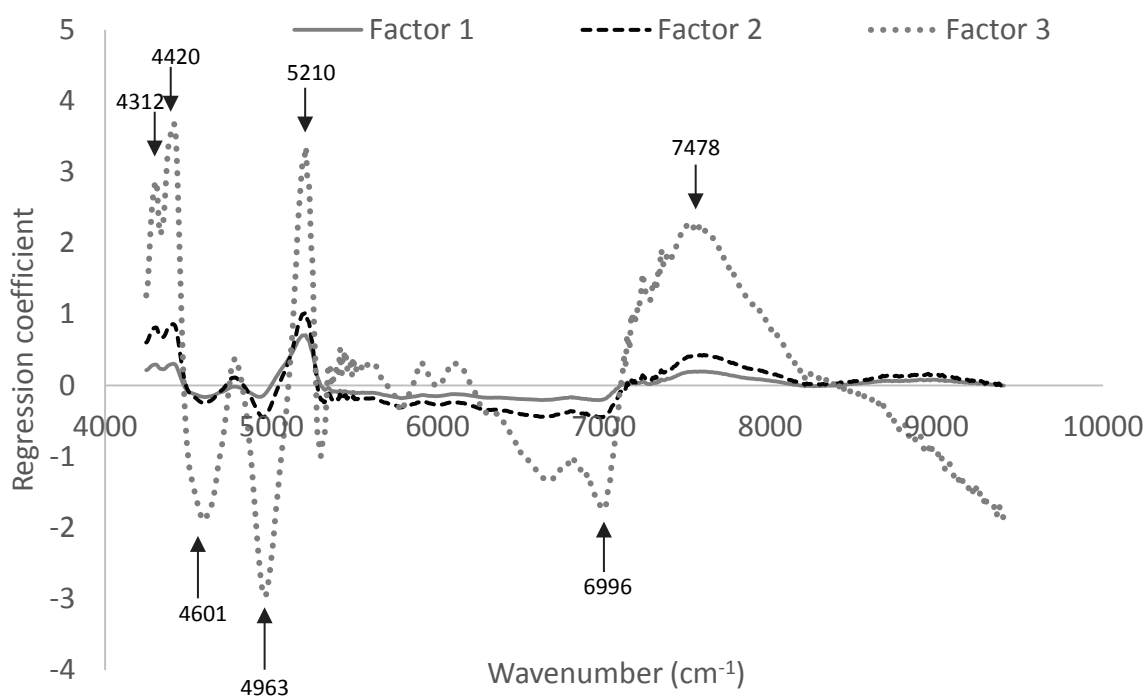
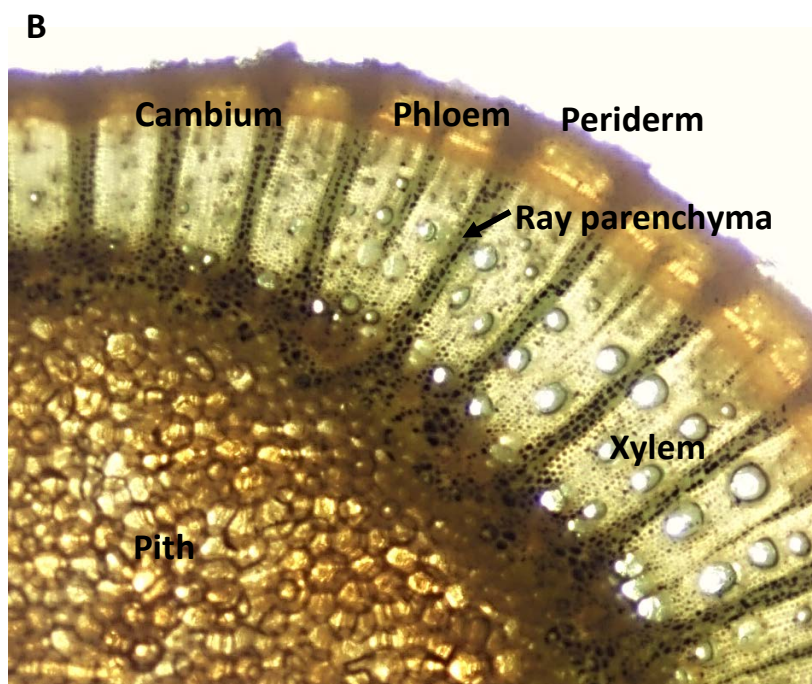
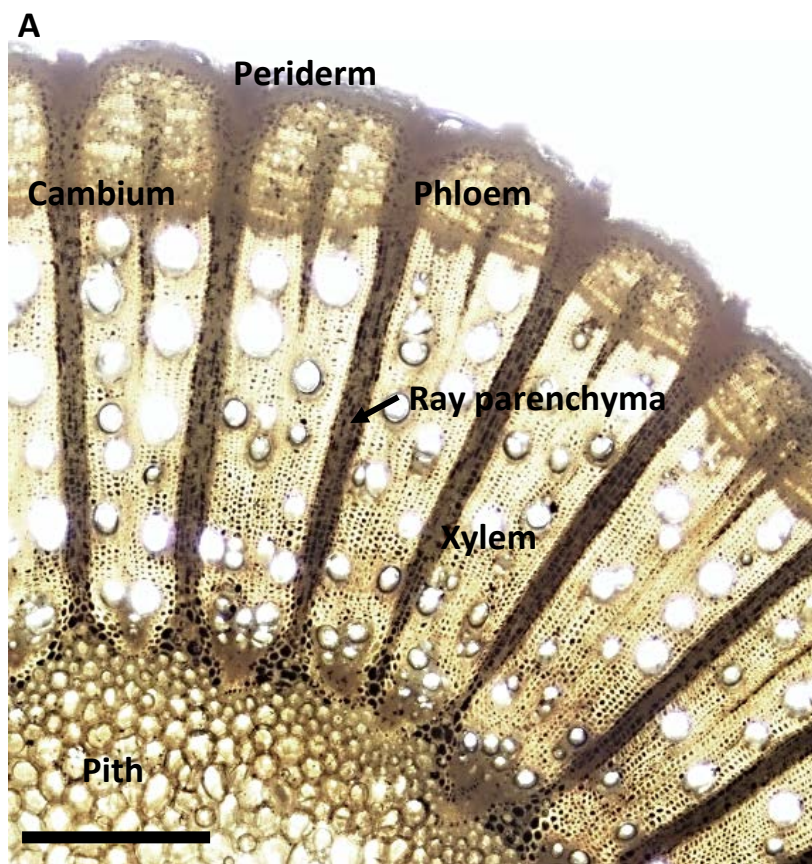


Figure 4. The first three partial least squares regression coefficients (Factors 1, 2 and 3) for the partial least square model developed to predict starch concentration (%) using the transformed NIR spectrum collected on ground cane wood (*Vitis vinifera*) obtained across four growing seasons (2010, 2011, 2012 and 2018).




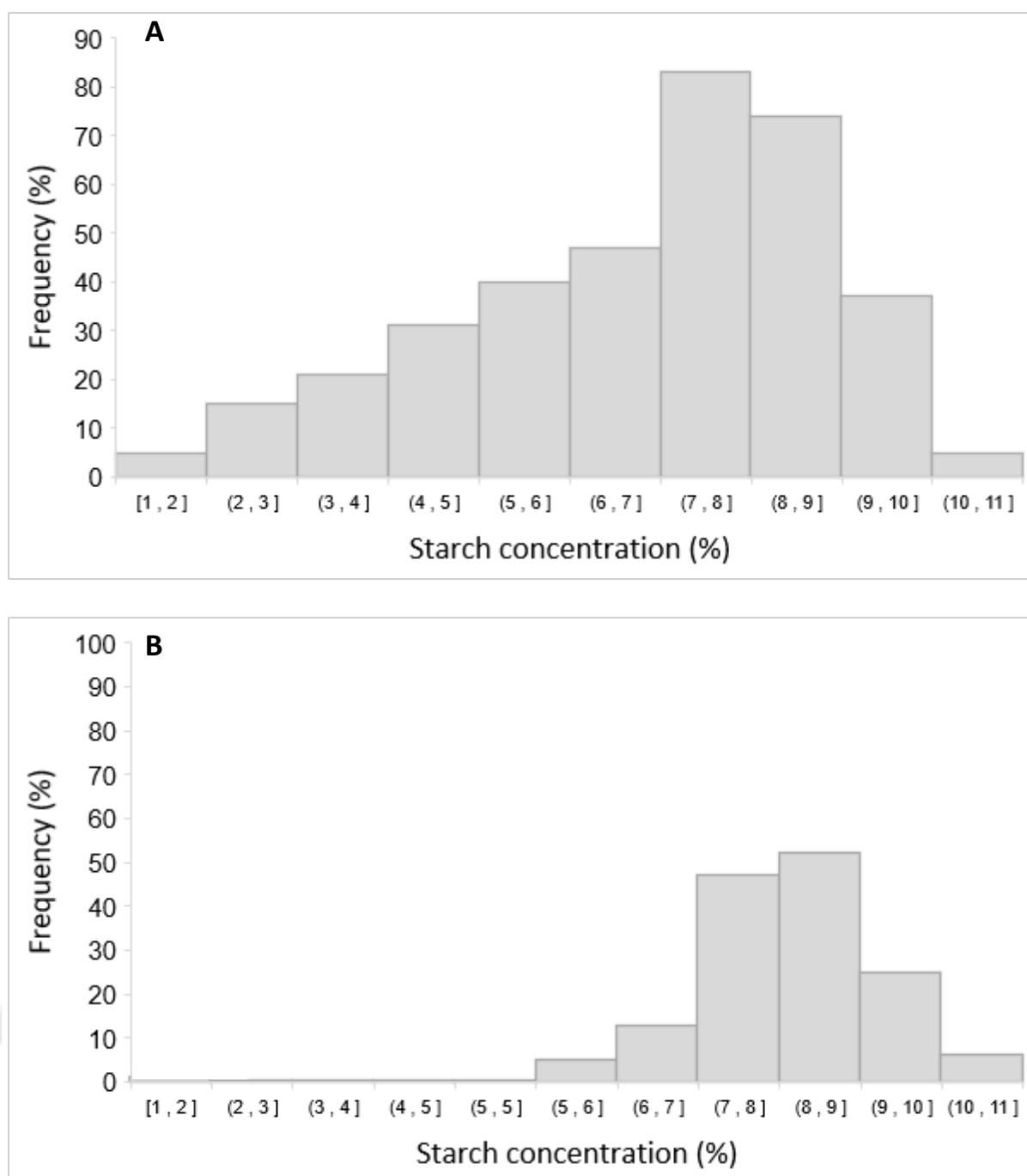


Figure 5. Light micrographs of transverse section, stained with 1% aqueous solution of iodine-potassium iodide, showing starch grains (arrows) in the ray parenchyma of Chardonnay (A) and Pinot Noir cane wood (B). Scale bar = 2 mm



Appendix 1. Histograms of starch concentration (%) of A) ground cane wood (*Vitis vinifera*) obtained across four growing seasons (2010, 2011, 2012 and 2018) and B) intact cane wood obtained in 2018.