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Exploration of global and specialized near infrared calibrations for the quantification of nutritional content in grapevine organs, berry phenological stages and shoot lignification.

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Abstract

Current infrared spectroscopy applications in the field of viticulture are moving towards direct in-field measuring techniques. However, limited research is available on quantitative applications using direct measurement on fresh tissue. The few studies conducted have combined the spectral data from various cultivars, growing regions, grapevine organs, and phenological stages during model development. The spectral data from these heterogeneous samples are combined into a single dataset and analysed jointly during quantitative analysis. Combining the spectral information of these diverse samples into a global dataset could be an unsuitable approach and could yield less accurate prediction results. Spectral differences among samples could be overlooked during model development and quantitative analysis. The development of specialised calibrations should be considered and could lead to more accurate quantitative analyses. This study explored a model optimisation strategy attempting global and specialised calibrations. Global calibrations, containing data from multiple organs, berry phenological and shoot lignification stages, were compared to specialised calibrations per organ or stage. The global calibration for organs contained data from shoots, leaves, and berries and produced moderately accurate prediction results for nitrogen, carbon, and hydrogen. The specialised calibrations per organ yielded more accurate calibrations with a coefficient of determination in validation (R^2val) at 90.65% and a root mean square error of prediction (RMSEP) at 0.32% dry matter (DM) for the berries' carbon calibrations. The leaves and shoots carbon calibrations had R^2val and RMSEP at 84.99%, 0.34% DM and 90.06%, 0.37% DM, respectively. The specialised calibrations for nitrogen and hydrogen showed similar improvements in prediction accuracy per organ. Specialised calibrations per phenological and lignification stage were also explored. Not all stages showed improvement, however, most stages had comparable or improved results for the specialised calibrations compared to the global calibrations containing all phenological or lignification stages. The results indicated that both global and specialised calibrations should be considered during model development to optimise prediction accuracy.

Keywords: viticulture, spectroscopy, chemometrics, nutritional content, specialized calibrations, global calibrations

Introduction

The popularisation of infrared spectroscopy technologies is leading to new viticultural applications to measure fresh grapevine material.[1–3] Viticultural samples are extremely diverse due to the chemical, morphological, and physical changes that occur in plant material throughout the growing stages such as vegetative growth, berry development, and dormancy.[4–6] Additionally, spectral information from multiple cultivars, growing sites, vintages, grapevine organs, and phenological stages could be included during sampling. This contributes to the number and heterogeneity of samples as well as the diversity of spectral data obtained. Infrared spectroscopies supply the means of rapidly measuring a diverse range of viticultural samples.

In current viticultural infrared applications, spectral data from heterogeneous samples are combined into a single dataset and analysed jointly during quantitative analysis.[6–8] Combining the spectral information of these diverse samples into a global dataset might not be the best approach and could yield less accurate prediction results. There could be spectral differences among cultivars, growing sites, vintages, grapevine organs, or phenological stages that are overlooked. The development of specialised calibrations should be considered and could lead to more accurate quantitative analyses.

Limited studies investigated the development of specialised calibrations per cultivar or grapevine organ, with mixed results.[9,10] One such study investigated two cultivars from one growing site and one vintage for the prediction of total soluble solids, total anthocyanins, and flavonoids.[10] A global calibration for both cultivars was developed in addition to prediction models for Shiraz and Cabernet Sauvignon. The global predictions for total soluble solids and total anthocyanins yielded very accurate and similar results, but no improvements in the calibrations per cultivar were observed. Furthermore, the global prediction for yellow flavonoids initially yielded poorer results, while per cultivar predictions showed a slight improvement.[10] It should be noted that two cultivars from a single site and vintage were included in the study and more complex datasets may yield different results.

Additionally, De Bei *et al.*[9] investigated the prediction of non-structural carbohydrates in the dried and ground trunk and leaf samples. Prediction accuracy for the global calibration, combining both organs, was moderate. Predictions per grapevine organ led to slightly poorer results for trunk samples and marginally more accurate results for leaves. Although both studies showed separation based on the phenological stage in principal component analysis (PCA),[9,10] the phenological stage was not considered during calibration development. These studies showed the feasibility of constructing specialised calibrations per cultivar or organ.[9,10] However, further exploration of calibrations per phenological stage should be considered.

Based on the above, our study proposes the exploration of global and specialised calibrations. Global calibrations consist of the entire sample set, while specialised calibrations contain a subset of the larger dataset. The use of specialised calibrations may improve accuracy when compared to global calibrations that combine data with varying spectral features. The reference analysis of carbon, hydrogen, nitrogen, and sulphur (CHNS) was selected for the exploration of prediction calibrations.

Information on the CHNS concentration in a vineyard could be indicative of its nutritional status. The nutritional status of the grapevine determines the growth and maintenance within a particular season and the subsequent grape composition and wine quality.[4,11,12] The grapevine's nitrogen concentration impact growth, grape quality, and carbon balance.[4] Additionally, vine growth, berry sugar accumulation, and anthocyanin biosynthesis are influenced by carbon concentrations.[8,13,14] Various important chemical reactions are affected by the hydrogen content, including respiration and carbohydrate accumulation. Furthermore, hydrogen is involved in the formation of multiple compounds, such as sugars, starch, amino acids, and organic acids.[15] The CHNS concentrations can additionally be used to supply information on the source-sink relationship between the grapevine organs.[4,8,13] The ability to measure and monitor nitrogen, carbon, and hydrogen concentrations could provide valuable information on the nutritional status of a vineyard throughout the growing season and aid fertilisation decisions.

Previously, extensive clustering analyses were performed using PCA and unsupervised self-organising maps (SOM).[16] Separation among shoots, leaves, and berries was reported for the grapevine organs. Furthermore, clustering emerged around the phenological and shoot lignification stages. However, no differences were observed among cultivars, growing sites, or vintages. Therefore, the main aim of this study is to further investigate specialised calibrations per grapevine organ, berry phenological stage, and shoot lignification stage as an alternative to current global calibrations combining the data.

Materials and Methods

Sample collection

Five grape producers and six growing sites in the Stellenbosch district of South Africa were used for sample collection. Table 1 summarises the GPS locations of the growing sites, the cultivars sampled at these locations, as well as the vintages. Some vineyard blocks were sampled over two vintages (2019-2020; 2020-2021) Fresh grapevine material was collected from ten vineyard blocks per vintage over the six growing sites from five grape producers during the above-mentioned vintages. Sampling started in November and ended in March for the respective vintages, with one additional sampling in July 2021 at dormancy. The vineyard blocks sampled consisted of seven cultivars including, Chardonnay, Sauvignon Blanc, Shiraz, Cabernet Sauvignon, Merlot, Malbec, and Pinotage. Five canes were sampled destructively between 06:00 and 10:00 from each block monthly and transported to the laboratory.

The intact canes were separated into shoots, leaves, and berries. Analysis was conducted and completed within 36 hours of sampling and relevant samples were stored at 4 °C overnight.[8] The modified Eichhorn-Lorenz system (EL) published by Coombe[17] was used to assign phenological stage for each vineyard block per month. Various phenological stages were sampled for each grapevine organ thoughout the growing season. Shoots were sampled between phenological growth stages ranging from EL15 (8 Leaves present) to the end of leaf fall (EL47). The berries' stages were from EL29 (Peppercorn-size) to EL39 (Over-ripe), and the leaves from EL15 (8 Leaves present) to EL41 (Cane maturity).[17] In total 3431 samples were collected with 1514 shoot

samples, 1540 leaf samples, and 476 berry samples.

Infrared spectroscopy analysis

Near infrared measurements were performed using a multi-purpose analyser (MPA) Fourier transform near infrared (FT-NIR) instrument (Bruker Optics, Ettlingen, Germany), fitted with a fibre-optic solid probe. The near infrared spectral range from 12000 to 4000 cm^{-1} was measured at a resolution of 4 cm^{-1} at 10 KHz. Shoots and leaves showed optimal spectra with reduced noise after 16 scans, however berries required 64 scans to reduce noise and increase spectral quality. The average of 16 scans was therefore obtained for shoots and leaves while 64 scans were average for berries. Canes were divided into top, middle, and bottom shoots sections of equal length. Shoots section above 35 cm were scanned four times, and sections below were measured twice, as repeats. Five anti-clockwise positions were scanned per leaf surface corresponding to leaf lobes. The first scan was at the top left basal lobe, followed by the left lateral lobe, the apical lobe, the right lateral lobe, and ending at the top right basal lobe. Whole bunches were destemmed and five representative berries per bunch selected for measurement in an upright position. A total number of 13827 spectra were collected. Two or four scans per shoot were collected depending on the length of the shoot with a total number of 3747 scans. Spectral measurements (7700) were obtained from the five lobes of each leaf. Finally, five berries were selected per cluster leading to 2380 berry scans.

Reference data

The reference data used in this study were obtained using in-house optimised PLS calibrations. Calibrations were developed for nitrogen, carbon, and hydrogen and for shoots, leaves, and berries. Eighty shoots, leaves, and berries samples were used to develop the calibrations. A total number of 240 samples was used. The average nitrogen content for the grapevine organs had a range between 0.14 and 3.71% dry matter (% DM), a mean of 1.24% DM and a standard deviation of 0.42% DM. The average carbon content had a range, mean and standard deviation of 39.10 to 48.96, 44.68, and 1.32 in % DM, respectively. Finally, the hydrogen reference data range was 5.56 to 8.03 % DM, the mean was 6.87 % DM, and the standard deviation was 0.44 % DM. The

values for nutritional content in the grapevine organs obtained were in line with other studies.[8] Various performance evaluation indices were used to evaluate the calibrations including coefficient of determination (R^2) and root mean square error of estimation (RMSEE) and prediction (RMSEP). The R^2 indicated the explained variance in the calibration (R^2cal) and validation (R^2val) datasets, while RMSEE and RMSEP indicated model prediction accuracy. The RMSEE and RMSEP was expressed in both percentage and units of measure. The calibrations for nitrogen content (% DM) had an average R^2cal and R^2val of 89.97% and 86.65%, and RMSEE (% RMSEE) and RMSEP (% RMSEP) of 0.15 (13.05%) and 0.14 (13.6%), respectively. The carbon calibrations also in % DM showed average R^2cal and R^2val of 77.24 and 66.75%, and RMSEE (% RMSEE) and RMSEP (% RMSEP) of 0.66 (1.47%) and 0.74 (1.65%). Finally, the hydrogen calibrations (% DM) presented average R^2cal and RMSEE (% RMSEE) of 81.22% and 0.19 (2.77%), and R^2val and RMSEP (% RMSEP) of 70.66% and 0.23 (3.25%), respectively. These calibrations were used to predict the nitrogen, carbon, and hydrogen content of the samples and spectral dataset used in this study (sections 2.1 and 2.2) to evaluate the suitability of global or specialised calibrations. Limited outlier removal was applied to the data when the predicted values differed with more than 0.2% DM below or above the minimum maximum range of the initial reference data.

Chemometric analysis

OPUS software (OPUS v. 7.2 for Microsoft, Bruker Optics, Ettlingen, Germany) was employed to perform partial least squares (PLS) regression. A manual dataset split of 1-5-5 was used. The fist number corresponds to the sample number the split sequence starts (number 1 in our case), the second number to the block length of test samples (5 samples were included in the validation set) and the third number to the block length of the calibration samples (5 samples). The reason for the specific split was that multiple spectra corresponded to one leaf, shoot, or berry sample. This dataset split was employed to ensure that different samples were used for calibration and validation, and not just spectral repeats. Fifty percent of the samples were included in the validation set.

Numerous spectral pre-processing algorithms were explored including constant offset elimination, straight line subtraction, standard normal variate (SNV) (also known as vector normalisation), min-max normalisation, multiplicative scattering correction (MSC), first derivative, and second derivative. The option of no spectral pre-processing was also examined for each calibration. Some combined pre-processing methods such as first derivative with straight line subtraction, first derivative with SNV, and first derivative with MSC were also investigated. The OPUS software initially uses the entire spectral region for optimisation, whereafter the regions identified during model validation are used individually for test set validation.

Rank determination

Two statistical approaches were employed during rank determinations using OPUS (OPUS v. 7.2 for Microsoft, Bruker Optics, Ettlingen, Germany) and R (version R i386 4.1.3) with RStudio (version 2021.09.0 Build 351) software. The OPUS software determines the rank (number of latent variables) from the predicted residual error sum of squares (PRESS) not significantly larger than the minimum. Once the minimum PRESS is found, the lower rank PRESS values and the minimum are used to calculate a proportional value. The proportional value is then used to calculate a probability and the optimal rank is selected as the first rank with a probability below 0.75.[18] The randomisation test used in R software uses a permutation approach that tests if adding additional variables is beneficial. It uses a backwards approach starting at the global minimum of the cross-validation curve. The algorithm continued to reduce the number of latent variables until significant deterioration of performance was observed (based in the $\alpha = 0.01$ level).[19,20]

Results and Discussion

The strategy proposed in this section is based on the reasoning that viticultural samples are extremely heterogeneous and change continuously throughout the growing season. These changes could be detected in their infrared spectral properties. The model optimisation strategy investigates the hypothesis that specialised calibrations per grapevine organ, phenological stage, or lignification might be more accurate than global calibrations combining samples. The benefits

of infrared applications include the ability to rapidly measure many samples. The large datasets provide a unique opportunity to investigate specialised calibrations that could lead to more individualised viticultural solutions. This section first discusses the rank determination approach for all the calibrations, followed by the assessment of the global and specialised calibrations per grapevine organ. Next, the berries dataset is investigated for global and specialised calibrations per phenological stage. Finally, the shoots dataset is explored for calibrations per lignification stage, comparing the global and specialised calibration results.

Rank determination

During PLS regression selecting the optimal rank or latent variables per calibration is a critical step.[18,20,21] However, choosing the optimal rank is often not a simple task and over-fitting needs to be prevented. Overfitting is defined as the selection of too many latent variables or too high a rank that describes random noise, in addition to the relationship between the calibrations and the validations.[21] The optimal rank is mostly selected based on the minimum RMSEP; however, the situation is more complex. An inverse relationship between the contribution of bias and variance to RMSEP is often found and a trade-off between the two needs to be considered. The variance increases with increasing model complexity, while the bias decreases. However, often the relationship is not entirely inverted because the increase in variance is slower than the decrease in bias. Thus, instead of leading to a minimum RMSEP where the variance and bias curves intersect, the RMSEP continues to decrease with increasing model complexity (rank).[20,21]

Two statistical approaches (PRESS and randomisation test) were investigated, as discussed in section 2.4.1, with the randomisation test proposed as an alternative to prevent over-fitting.[19,20] Comparable results were obtained for both approaches. Initially, ranks between 12 and 15 were explored since these ranks are widely reported in the literature.[9,18,22–24] Rank fifteen was always reported as the optimal option with both the PRESS and randomisation test approaches. In addition to this, the calibrations generated between ranks 12 and 15 often reported near-perfect R^2 values of close to 100%. The results could still be attributed to overfitting, especially since the datasets used consisted of large numbers of samples (between 2380 and 7701).

After further investigation of the literature, it was decided to adopt a conservative approach and explore ranks between 5 and 8.[6,7,21] Deng *et al.*[21] suggested the use of ranks 6 to 8 for infrared applications of chemical modelling. However, certain calibrations showed rank five to perform better than rank six and were thus included. Both statistical approaches for rank determination (PRESS and randomisation test) showed an optimal rank of eight for most datasets, while a rank of seven was also reported for a few datasets. However, when evaluating the calibration statistics rank eight again often yielded near-perfect R^2 values not normally reported for infrared applications. Therefore, to eliminate the possibility of overfitting and to select the best possible model that accurately and truthfully represents the data, ranks between 5 and 8 were selected for models showing reasonable prediction accuracy with R^2 values below 95%.

Calibrations per grapevine organ

The global calibrations explored in this section include data on the three organs (shoots, leaves, and berries), while the specialised calibrations per organ were also investigated. The calibration statistics are summarised in Table 2. The global calibrations included 13828 datapoints, while the shoots dataset contained 3747, the leaves 7701, and the berries 2380. The calibration and validation split for each dataset is also shown in Table 2 with the percentage outliers removed (%OR). The global calibration for nitrogen showed R^2cal and R^2val values of 88.47% and 88.76%, respectively. The RMSEE and RMSEP were below 17%. The prediction of nitrogen per grapevine organ showed improved results from the global prediction. The nitrogen calibration for the berries yielded R^2cal and R^2val of 93.64% and 92.66%, respectively, and RMSEE of 11.60%, and RMSEP of 12.86%. Similarly, accurate results were found for the leaves' nitrogen calibration with R^2cal at 91.92%, R^2val at 91.79%, RMSEE at 5.95% and RMSEP at 5.85%. The calibration for the shoots nitrogen reported slightly improved results with R^2cal of 93.98%, R^2val of 92.85%, RMSEE of 8.06%, and RMSEP of 8.77%. Accurate predictions were observed for all three organs for the nitrogen calibrations compared to the global calibration.

The global model for carbon prediction showed moderate accuracy with R^2cal of 57.77% and R^2val of 55.13%. Despite this results, very low RMSE values were obtained for the RMSEE

(1.61%) and the RMSEP (1.68%). The carbon calibrations per grapevine organ showed significant improvement. The highest prediction was reported for the berries carbon calibration with R^2cal at 94.60%, R^2val at 90.65%, RMSEE at 0.50% and RMSEP at 0.71%. A slight decrease in predictability was seen for the leaves carbon calibration compared to the berries' calibration, although a marked improvements was still observed compared to the global calibration. The R^2cal and R^2val was 86.39% and 84.99%, respectively, with RMSE below 0.8%. The carbon calibrations for the shoots reported R^2cal of 91.26%, R^2val of 90.06% and RMSE close to 0.8%. Overall, the carbon predictability was highly accurate for all three organs. A representation of the observed versus predicted graphs for the carbon calibrations is shown in Figure 1.

The hydrogen calibrations showed a similar trend with the global calibration yielding less accurate models. R^2cal of 74.01% and R^2val of 68.42% were reported for the global hydrogen calibration with RMSEE of 3.23% and RMSEP of 3.54%. The berries hydrogen calibration displayed improved accuracy with R^2cal at 92.72%, R^2val at 89.01%, RMSEE at 1.97% and RMSEP at 2.54%. Further increased accuracy was seen for the hydrogen calibration of the leaves with R^2cal at 95.22% and R^2val and 94.83% and RMSE below 1.2%. The least marked improvement was observed for the shoot's hydrogen calibration of the organs, however, an improvement was still seen. The R^2cal and R^2val was 80.53% and 75.22%, respectively, the RMSEE was 1.60% and the RMSEP was 1.84%. Overall, the hydrogen calibrations per grapevine organ similarly had increased predictability compared to the global calibration.

The specialised calibrations per organ showed more accurate predictability for nitrogen, carbon, and hydrogen calibrations than previously reported in literature for fresh grapevine organs.[8] Cuq *et al.*[8] reported R^2cal of 90%, R^2val of 84%. Carbon predictions were reported at 67% for R^2cal and 57% for R^2val. The hydrogen calibrations reported poor predictability with R^2cal and R^2val at 54% and 50%, respectively.[8] Table 2 show the optimal pre-processing methods used for each calibration. Similar pre-processing methods were seen for the nitrogen and carbon calibrations, including straight line subtraction and first derivative. The hydrogen calibrations reported the optimal results when a combination of pre-processing methods was used. First derivative pre-processing together with SNV and MSC was used, as well as straight line subtraction. The same

pre-processing methods were used in literature by similar studies, including first derivative and MSC.[8,10]

To investigate the spectral difference between the grapevine organs the average raw spectra were calculated and compared. Although differences were observed, pre-processing was employed to highlight the differences and the relevant spectral bands. Various pre-processing methods were explored, but first-order Savitzky-Golay spectral derivatives showed the most pronounced distinctions between the spectra. The algorithm initially smooths the spectra and emphasises the small bands and resolves overlapping peaks.[8,25] A similar approach was used to highlight the spectral differences between grapevine organs by Cuq *et al.*[8] The pre-processed spectra for each grapevine organ are shown in Figure 2, with the spectral regions used for the calibrations of nitrogen, carbon, and hydrogen. The spectral regions, as well as the areas they overlap, are visually presented in Figure 2.

The relevant absorption bands shown in Figure 2 for the grapevine organs were from 4100 to 5700, 6800 to 7400, 8500 to 9000, and 10200 to 10500 cm^{-1}. Our previous study, using orthogonal partial least squares discriminant analysis (OPLS-DA) and S-plots, identified three spectral regions of interest between the grapevine shoots and leaves identified (5115 – 5240, 8830 – 9800, and 10600 – 11300 cm^{-1}).[16] Similar regions were observed for fresh grapevine leaves and berries in literature.[8] The same regions are observed in Figure 2 with prominent differences from 5115 to 5240 cm^{-1} and minor differences between 8830 to 9800 and 10600 to 11300 cm^{-1}.

In Table 2 and Figure 1 an additional region can be seen for the global nitrogen calibration at 4000 to 4850 cm^{-1}. This region has been associated with nitrogen in a variety of plant leaves.[26] Markedly, the most noticeable peaks are in the region between 4100 and 5500 cm^{-1}, but this region was only used in the berries' hydrogen calibrations. The 5200 cm^{-1} absorption band have been associated in literature with the OH first overtones and O-H stretching/HOH deformation of water.[6,9,27] The connection of the larger peaks with water could explain the differences between the organs and why that region was used during hydrogen calibrations.

Although specialised models per organ were constructed, these calibrations still include various

cultivars, growing regions, and vintages. A substantial improvement in the predictions of nitrogen, carbon, and hydrogen was seen for the calibration models constructed per grapevine organ compared to the global calibration combining all organs. This validates our hypothesis that constructing specialised calibrations could improve prediction accuracy. The range of reference compounds differed for each organ and the specialised calibrations could be applied to monitor these compounds per organ during the growing season. The information obtained could be used for specialised viticultural decisions based on the nutritional status of the vineyards. Important knowledge on the source-sink relationship between the organs could be obtained as well as information on the nitrogen and carbon accumulation per organ.[4,5,8,13] If decreases are seen specialised fertilisation solutions per organ could be considered such as foliar or bunch applications.

Calibrations per phenological stage for berries

The berries dataset was explored for specialised calibrations per phenological stages. However, eight phenological stages were initially assigned during berry sampling[17] and some stages consisted of very few spectra (Table 3). The unsupervised SOM in our previous study showed the grouping of certain phenological stages.[16] Stages were grouped based on previous findings and sample size. The phenological stages EL29 and 31 were combined to represent berries between 4 and 7mm in diameter, and EL37 to EL39 were grouped to represent ripe grapes. Finally, five phenological stages were investigated in specialised models, including EL29-31, EL32, EL33, EL35, and EL37-39 as shown in Table 3.

Global calibrations included all phenological stages, while the specialised calibrations contained one phenological stage at a time as shown in Table 3. The prediction results for the global and specialised calibrations per reference compound are summarised in Table 4. The calibrations for nitrogen, carbon, and hydrogen for the berries dataset have been previously reported in Table 2 but are shown again in Table 4 as the global calibrations. The berries global nitrogen calibration was shown to be accurate with R^2 values above 92% and RMSEE values between 11 and 13%. The calibrations for EL29-31 and EL32 showed increased R^2 values above 94% and much lower

RMSE below 3.2%. The EL33 calibration showed similar results to the global calibration although the RMSEE values were still much lower. The calibrations for EL35 and EL397-39 showed a slight decrease in R^2 values, but RMSE values were comparable to the global calibration.

The global and specialised calibrations for carbon were more similar with only the EL33 calibration showing slightly higher R^2cal (97.45%) and R^2val (92.09%) than the global calibration. The RMSE values were comparable across all models. However, all reported carbon calibrations showed accurate predictability with R^2 values ranging from 80 to 98% and showed that the global, as well as specialised models, can be used for carbon determination. The global hydrogen calibration was only outperformed by the EL29-31 calibration which reported R^2cal and R^2val of 95.84 and 92.34%, respectively. The EL33 and EL35 calibration showed comparable results, while EL32 and EL37-39 showed poorer validation results (R^2val 72 – 80%) compared to the global hydrogen calibration. However, overall, the hydrogen models show good predictability with R^2 values above 80% and low RMSE.

The pre-processing method of choice seemed to differ per reference compound, although there was some overlapping. The nitrogen calibrations used a few pre-processing methods, including straight line subtraction, SNV, and first derivate with SNV. The carbon calibration only made use of straight-line subtraction. The hydrogen calibrations mostly used MSC, although straight line subtraction and first derivative with SNV were also seen.

Figure 3 shows the pre-processed average spectra (first derivative Savitzky-Golay) per phenological stage with the spectral regions used during calibration per reference compound (Table 4). Less and smaller spectral regions seem to be shown for the reference compounds compared to the per organ calibrations (Figure 2). This could indicate that more specific spectral regions associate with the phenological stages, rather than almost the whole spectra as were seen per grapevine organ. The regions reported show abundant overlapping between the reference compounds with one unique region for the hydrogen calibration (4850 – 5700 cm^{-1}) once again seen around the spectral band possibly associated with water.[6,9,27]

Although the pre-processed spectra per phenological stage look similar, subtle differences and

changes throughout the growing season can be observed. The regions between 4000 and 7400 cm^{-1} seem to show the most variation with small differences between 8500 to 9000 cm^{-1} and 10200 to 10500 cm^{-1}. Similar regions (6900 – 7400, 8300 – 9100, 10250 – 10950 cm^{-1}) were reported in the literature for fresh berries and leaves.[8] Additionally, in our previous study the 4500 to 5300 cm^{-1} and 6000 to 7300 cm^{-1} regions were associated with fresh shoots, leaves, and berries, while the 8000 to 8700 cm^{-1} and 9500 to 10500 cm^{-1} regions corresponded to berries.

A recent study investigating the amino acid content in fresh grape berries also found important spectral regions between 5260 to 5300 cm^{-1} and 8700 to 8900 cm^{-1}. The first region (5260 – 5300 cm^{-1}) was also associated with the stretching and deformation of the OH group in water, while the second region (8700 to 8900 cm^{-1}) was linked to the second overtones of C-H stretching of aromatic structures.[6] Some literature investigated the spectral regions associated with the differences in ripening stages of grapevine berries.[10,27] Two regions were identified between 4850 to 5700 cm^{-1} and at 10000 cm^{-1} and were related to the first and second overtones of sugar and water absorption bands (5070 – 5260 cm^{-1}).[10,27]

The monitoring of nitrogen, carbon, and hydrogen at specific phenological stages throughout the growing season could greatly aid viticultural decisions regarding fertilisation. Additionally, this information could improve our knowledge of nutrient movement and deficiencies over the growing season. These compounds all play a key role during berry development and their requirements can change according to the developmental stage.[4,13] While lower nitrogen before véraison could benefit berry development, nitrogen restraints after véraison could be detrimental to berry enlargement. Nitrogen content also influences the amino acid and aromatic precursors in grape berries and impacts grape quality.[4] The applications of fertilisers could increase nitrogen availability, influence carbon partitioning, and improve the uptake of carbon and nitrogen in the grapevines. There is a delicate balance between nitrogen addition and carbon assimilation[4,8,13] and the calibrations could be used to optimise viticultural decisions. Nutrient applications at the optimal time during the growing season could increase grape quality and improve ripening.

Calibrations per lignification for shoots

399 Global calibrations included all lignification stages of the shoots, while specialised calibrations
400 evaluated each lignification stage separately. The global calibrations were previously reported in
401 Table 2, but are included again in Table 5, together with the specialised calibrations. A total of
402 3747 spectra were included in the global calibrations, which split into 1938 for the unlignified,
403 1541 for the lignified, and 268 for the dormant datasets. Shoot lignification was assigned based
404 on a visual assessment of the shoots. Unlignified shoots were fresh and green in colour, while
405 lignified shoots were brown and dry. Dormant samples were collected for one month (July) in the
406 second vintage at the phenological stage EL47 after leaf fall.

407 The global shoots' calibrations for nitrogen showed very accurate prediction results with R^2 values
408 above 92% and RMSE between 8 and 9%. The unlignifed calibration for shoot nitrogen was the
409 only calibration that showed better predictability with R^2cal of 95.65%, R^2val of 94.34%, RMSEE
410 of 5.98% and RMSEP of 7.61%. The lignified calibration showed R^2 values below 82%. The
411 dormant calibrations showed a further decrease in predictability with R^2 values below 72%. The
412 obsereved versus predicted graphs for the validation results are shown in Figure 4.

413 The carbon prediction for the global calibration initially showed accurate results with R^2 values
414 above 90% and RMSE below 1%. The unlignified calibration reported R^2cal of 97.36%, R^2val of
415 97.28% and RMSE below 0.5%. Once again, the unlignified calibration for carbon was the only
416 one that outperformed the global calibration. However, the lignified and dormant calibrations were
417 still accurate and comparable. The lignified carbon calibration showed R^2cal and R^2val of 91.02%
418 and 87.44%, respectively, with RMSE values at 0.5% and below. The R^2cal for the dormant
419 calibration was still at 90.90%, however, the R^2val was at 85.78%. The RMSE values remained
420 below 1%.

421 The global hydrogen calibration yielded average results with R^2cal of 80.53%, R^2val of 75.22%
422 and RMSE below 2%. Improved results were seen for the unlignified hydrogen calibration of R^2cal
423 and R^2val of 87.09% and 84.19%, respectively. The RMSE values remained low (1.5 and below).
424 The lignified calibration showed a slight further increase in R^2cal (88.95%), however, the R^2val
425 decreased marginally (82.58%), but was still higher than the global calibration. The RMSE values

decreased below 1.4%. The dormant calibration showed the most improvement with R^2cal at 95.46%, R^2val at 87.61% and RMSE at 1.5% and below. The obsereved versus predicted graphs for the validation results are shown in Figure 5.

Overall, only the hydrogen calibrations showed improvement for all three lignification stages compared to the global calibration. The unlignified nitrogen and carbon calibrations both showed increased predictability to the global calibration. The lignified and dormant nitrogen calibrations showed less accurate results, while the carbon calibrations for the lignified and dormant stages were comparable to the global calibration. The allocation of the lignification stage was done visually and due to the progressive nature of lignification, there could have been overlapping between the stages. The overlapping could have caused decreased predictability of certain specialised calibrations. However, these stages are still linked to important phenological and morphological changes in the shoots throughout the growing season and could supply valuable viticultural information.

The average pre-processed spectra (first derivative Savitzky-Golay) per lignification stage, as well as the spectral regions associated with each reference compound are shown in Figure 6. More specific and fewer spectral regions were observed for the reference compounds for the lignification stage than were seen for grapevine organs (Figure 2) and phenological stages (Figure 3). Although these regions still overlapped with the regions previously reported for the grapevine organ and phenological stage. A similar region between 5700 and 6550 cm^{-1} was observed for nitrogen and carbon, while the region between 7400 and 9950 cm^{-1} was associated with all three reference compounds. An additional region between 9950 and 10800 cm^{-1} was shown for carbon.

OPLS-DA and S-plot were used to identify the spectral regions of interest between shoots and leaves in our previous study.[16] Three spectral regions were observed (5115 – 5240, 8830 – 9800, and 10600 – 11300 cm^{-1}) corresponding to the regions reported for the reference compound calibrations (Figure 6). Additionally, the lignified and unlignified stages were investigated and the S-plot showed the regions between 4000 and 7400 cm^{-1} to be of interest. Similar results were described in the literature for dried and ground shoot samples with regions between 4000 and

8000 cm^{-1} reported and related to the vibrational signals of starch.[28] Additionally, De Bei *et al.*[9] observed absorption bands at 4300, 5200, and 7000 cm^{-1} for dried trunk samples and associated these regions in the spectra with protein, nitrogen, cellulose, and starch.[9] The absorption band associated with water was once again seen in the averaged spectra between 5000 and 5200 cm^{-1}.[6,27] The unlignified spectra show a much higher absorption band than the lignified and dormant spectra, as would be expected. However, this spectral band was not included during calibration development.

The mobilisation and movement of nitrogen and carbon in grapevines are constantly changing between different growing stages.[4,7] Additionally, hydrogen contributes to respiration reactions that can change even more rapidly.[15] The ability to predict these compounds at specific growing stages could yield valuable information on the movement of resources as well as the growth dynamics within the grapevines. The relationship between the source (leaves and shoots) and sink (berries) changes continuously depending on the growing stage and fertilisation supplies the means to manage this relationship.[4,11,13]

During the unlignified shoot stage when vegetative growth is extremely important, deficiencies in nitrogen and carbon could be detected and aid fertilisation additions. The lignified stage corresponds to the time when sufficient nitrogen and carbon reserves need to be accumulated. Insufficient reserves could negatively affect subsequent growing seasons.[7] However, a fine balance needs to be maintained to ensure optimal berry development and reserve accumulation.[4,13] Furthermore, the nitrogen and carbon concentrations in dormant shoot samples could be an early indication of growth and yield in the subsequent season.[7] The ability to apply fertilisation as needed at the optimal shoot growth stage could improve grapevine growth, health, yield, and improve these factors for following seasons.

Conclusion

The specialised calibrations for grapevine shoots, leaves, and berries yielded more accurate results than a global calibration combining all three organs, and these results were observed for nitrogen, carbon, and hydrogen calibrations. Moreover, for the specialised calibrations per

phenological stage in berries, some stages showed improvements, while others showed slightly less accurate results. Although all stages did not outperform the global calibration, all calibrations showed the ability to accurately predict the three reference compounds. The fact that the phenological stages are a combination of various cultivars, growing sites, and vintages makes them robust and demonstrates their possible application to a wide range of future samples. Finally, the performance of the global shoot calibrations compared to calibrations per lignification stage was explored. The unlignified calibrations showed increased predictability compared to the global calibration, while the lignified and dormant calibrations showed less or comparable accuracy. The exception was the hydrogen calibrations where all three lignification stages showed improved predictability. The global shoot calibration initially showed very accurate results, possibly making further improvements unlikely.

Although the calibrations per organ, phenological stage, and lignification for nitrogen, carbon, and hydrogen were discussed separately, all information obtained should be considered collectively. The knowledge of the concentration of these compounds in grapevine organs at specific growth stages could improve our interpretation of the source-sink relationship[4,8,13] and lead to more specific and judicious decisions regarding fertilisation applications. Increased pressure on viticulturists is forcing the use of less resources to obtain optimal grape quality.[11] The information obtained from our calibrations could lead to the sustainable and targeted applications of fertilisation at specific phenological and physiological stages. This information could facilitate the management of grapevine growth and the influence on resource participation from source to sink by viticulturists.[4,7,13] Improving the management of resources in the vineyard could enhance berry development, leading to increase amino acid and aromatic precursor content, and optimising grape and must quality.[4,12]

Although not all specialised calibrations showed more accurate prediction results, most showed an improvement to the global calibrations validating their consideration during model development. In future infrared spectroscopy applications, specialised calibrations should be considered, especially for diverse and heterogeneous agricultural and viticultural samples. The

calibration information could aid management decisions, facilitate the implementation of precision viticulture, and improve resource practices.

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588 **Tables and figures captions**

589 **Table I.** GPS locations of growing sites with cultivars sampled during each vintage.

590 **Table II.** Global and specialised calibrations per reference compound (nitrogen, carbon,
591 hydrogen) and per grapevine organ (berries, leaves, shoots).

592 **Table III.** Original and grouped number of spectra per phenological stage used in the calibrations

593 **Table IV.** Global and specialised calibrations per reference compound (nitrogen, carbon,
594 hydrogen) and phenological stage for the berries’ dataset.

595 **Table V.** Global and specialised calibrations per reference compound (nitrogen, carbon,
596 hydrogen) and lignification stage for the shoots’ dataset.

597 **Fig 1**. Validation results showing the observed (*y* axis) versus predicted (*x* axis) carbon content
598 as % dry mater (%DM) for (a) the global calibrations including all three organs (R2val = 55.13;
599 RMSEP = 0.75 %DM), (b) berries’ calibration (R2val = 90.65; RMSEP = 0.32 %DM), (c) leaves’
600 calibration R2val = 84.99; RMSEP = 0.34 %DM) and (d) shoots’ calibration R2val = 90.06;
601 RMSEP = 0.37 %DM). Increased prediction accuracy is observed in the per organ specialized
602 calibrations.

603 **Fig 2.** Average pre-processed spectra for berries (dotted line), leaves (dashed line), and shoots
604 (solid line) with spectral regions used for each calibration of nitrogen (a), carbon (b), and hydrogen
605 (c).

606 **Fig 3**. Average pre-processed spectra per phenological stage for berries’ dataset with spectral
607 regions used for each calibration of nitrogen (a), carbon (b), and hydrogen (c). EL29-31 –
608 Peppercorn-size to Pea-size (round dotted line), EL32 – Bunch closure (square dotted line), EL33
609 – Hard-green (dashed line), EL35 – Véraison (long dashed and dotted line), EL37-39 – Almost-

610 ripe, Harvest, and Over-ripe (solid line).

611 **Fig 4**. Validation results showing the observed (*y* axis) versus predicted (*x* axis) nitrogen (*N*)
612 content as % dry mater (%DM) for (a) the global calibration including unlignified, lignified and
613 dormant shoots (R2val = 92.85; RMSEP = 0.06 %DM), (b) unlignified shoots calibration (R2val =
614 94.34; RMSEP = 0.05 %DM), (c) lignified shoots calibration R2val = 78.36; RMSEP = 0.04 %DM)
615 and (d) dormant shoots calibration (R2val = 71.98; RMSEP = 0.06 %DM). Prediction accuracy
616 does not always increase for the specialized calibrations.

617 **Fig 5.** Validation results showing the observed (*y* axis) versus predicted (*x* axis) hydrogen (*H*)
618 content as % dry mater (%DM) for (a) the global calibration including unlignified, lignified and
619 dormant shoots (R2val = 75.22; RMSEP = 0.13 %DM), (b) unlignified shoots calibration (R2val =
620 84.19; RMSEP = 0.10 %DM), (c) lignified shoots calibration R2val = 82.58; RMSEP = 0.09 %DM)
621 and (d) dormant shoots calibration (R2val = 87.61; RMSEP = 0.10 %DM). Prediction accuracy
622 increases for the specialized calibrations.

623 **Fig 6.** Average pre-processed spectra for unlignified (dotted line), lignified (dashed line), and
624 dormant (solid line) shoots with spectral regions used for each calibration of nitrogen (a), carbon
625 (b), and hydrogen (c).