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Additional Information

The use of non-invasive fluorescence spectroscopy to quantify phenolic content under red wine real-time fermentation conditions

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ABSTRACT

Phenolic compounds play important roles in wine quality attributes such as colour, mouthfeel and ageing potential. The ability to monitor their extraction and implement appropriate vinification techniques relies on accurate phenolic analysis methods. Front-face fluorescence spectroscopy presents itself as a user-friendly, rapid and cost-effective alternative to other spectrophotometric methods. The main aim of the study was therefore to investigate the potential of fluorescence spectroscopy to directly measure phenolic content of red wine samples throughout red wine fermentations. Cabernet Sauvignon fermentations were monitored using fluorescence spectroscopy and UV-Visible spectrophotometry. Fermentation conditions were explored for their influence on the prediction accuracy of fluorescence-based regression models. The coefficient of correlation (R^2val) and root mean square errors of validation (RMSEV) for models built using non-invasive spectral data obtained from unaltered samples were 0.96, 0.94 and 0.89 and 1.38 index units, 42.84 mg/L and 20.53 mg/L for total phenols (index units (IU)), total condensed tannins (mg/L), total anthocyanins (mg/L), respectively. Overall, the ability to obtain high quality spectra from unaltered samples simulating direct measurements from the fermentation vessel was demonstrated and holds potential for on-line automated systems or portable device applications.

KEYWORDS

Fluorescence spectra, direct measurements, unaltered samples, phenolic compounds, chemometrics, machine learning

1. INTRODUCTION

Red wine production involves alcoholic fermentation taking place in the presence of both solid and liquid phases of the grape must, resulting in the suspension of grape solids, yeast and various colloidal particles. Phenolic extraction relies on adequate skin-juice contact and various winemaking techniques implemented pre-, post- or during fermentation have been studied for their influence on the resulting red wine phenolic profile (Casassa & Harbertson, 2014; Sacchi et al., 2005; Smith et al., 2015). These vinification techniques may include the addition of pectolytic enzymes, cap management in the form of pump-overs or punch-downs as well as extended maceration, among others (Sacchi et al., 2005). Anthocyanins, flavonols and their subsequently polymerised forms are considered to have the greatest sensory impact on red wine, specifically with regards to important attributes such as mouthfeel, colour and ageing potential (Sacchi et al., 2005).

Anthocyanin extraction reaches a maximum early on in the fermentation followed by a decline thereafter because of co-pigmentation and polymerisation reactions, while condensed tannins experience continued skin-juice extraction with seed tannins increasing linearly compared to the earlier plateau reached by skin tannins (Cadot et al., 2006; Canals et al., 2005; Sacchi et al., 2005). Understanding the extraction dynamics of phenolic compounds may aid in implementing timely winemaking practices for the desired effect and therefore requires the routine analysis of these important compounds throughout fermentation. The benefits of fluorescence spectroscopy, including its non-invasive technique, increased sensitivity, rapid and user-friendly action as well as its relative cost-effectiveness when compared to other time consuming conventional spectrophotometric methods that require a sample preparation step, measurement of a specific wavelength and quantification with a

standard compound (Aleixandre-Tudo et al., 2017). This has allowed fluorescence spectroscopy to become an increasingly popular alternative in various food science disciplines (Airado-Rodríguez et al., 2011; Karoui & Blecker, 2011; Strasburg & Ludescher, 1995). Front-face fluorescence spectroscopy is explored in this study as an alternative to the current spectrophotometric analysis methods used for phenolic analysis.

Understanding fluorescence spectroscopy instrumentation and the factors affecting optimal analysis are essential for collecting accurate and representative spectral information. The electronic transitions taking place during fluorescence data acquisition, namely the absorption of UV-Visible light, the subsequent redistribution of energy by excited electrons within fluorescent compounds and their detected emitted light, are influenced by several factors such as quenching, the local environment and light scatter phenomena (Karoui & Blecker, 2011; Strasburg & Ludescher, 1995). Higher temperatures during analysis may increase collisional velocity and therefore collisional quenching, resulting in a decreased fluorescence intensity. The local environment including pH changes and sample colour influence the highly sensitive fluorophores, thereby influencing the shape and intensity of the captured fluorescence spectra, and light scatter phenomena such as Rayleigh scattering can be considerably affected in turbid or opaque samples with regards to the optical sampling depth as well as the captured fluorescence signal. The results from analysing diluted samples are not always comparable with those of the original sample, specifically with the matrix of food products significantly affecting intrinsic fluorescent compounds (Airado-Rodríguez et al., 2011). The sample geometry of front-face fluorescence eliminates the need for sample dilution as with conventional right-angle fluorescence and allows for the analysis of native samples (turbid, concentrated or solid) owing to the signal captured being independent of the light penetration through the sample (Airado-Rodríguez et al., 2011; Karoui & Blecker, 2011).

The minimal to no sample preparation required for this technique therefore holds potential for analysing red wine throughout fermentation directly from the fermentation vessel, an application which may be of benefit to the producer in on-line systems or portable devices.

The aim of this study was to investigate the prediction accuracy of five phenolic content regression models built using front-face fluorescence spectroscopy, while exploring the effects of fermentation conditions. The influence of carbon dioxide and turbidity caused by the presence of grape solids, and therefore the required sample preparation, was assessed in order to successfully analyse red wine samples throughout fermentation directly from the fermentation vessel. To the authors best knowledge, this is the first attempt to obtain fluorescence spectra from undiluted (no sample preparation) and unaltered (with solids in suspension and carbon dioxide) samples. Spectroscopy calibrations were then attempted to simulate direct measurements of phenolic content during the red wine fermentation process.

2. MATERIALS AND METHODS

2.1. *Reagents*. Ammonium sulphate, hydrochloric acid (HCl 1 M), methyl cellulose, sulphur dioxide (SO_2), ethanol (96%) and sodium metabisulfite (2.5 %) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany).

2.2. *Experimental design*. This study was performed using Cabernet Sauvignon grapes from the same vineyard harvested in the 2020 vintage and frozen in a -20 °C room until processing in the experimental cellar at the Department of Viticulture and Oenology (Stellenbosch University). Twenty kg of grapes were crushed and destemmed into a 20 L plastic bucket and received 50 mg/L sulphur dioxide (SO_2). The must was inoculated with 20 g/hL Zymaflore RX60 (Saccharomyces cerevisiae, Laffort, Bordeaux, France) and the fermentation took place in a 25 °C temperature-controlled room. Two punch-downs were performed per day. Sample

collection and analysis took place from the first day of fermentation until the wines had fermented dry (residual sugar < 4 g/L) 12 days later. Although sampled on consecutive fermentation days and UV-Vis spectrophotometric methods conducted daily, fluorescence analysis was performed only on 9 of those days due to logistical reasons.

Following the morning punch-down and homogenous mixing, three 15 ml test tube samples were collected per treatment. Three sample preparation treatments were investigated, namely clean samples (Treatment A), degassed samples (Treatment B) and unaltered samples (Treatment C). For each ferment, the first tube (Treatment A) was degassed by vacuum followed by centrifuging at 5000 rpm for 2 min in an Eppendorf 5415D centrifuge (Hamburg, Germany) and subsequently removing the supernatant to inhibit interference of fermentation sediment such as yeast and grape solids. The second tube (Treatment B) was degassed by vacuum to remove the carbon dioxide (CO_2) within the sample while remaining turbid, and the third tube (Treatment C) received no sample preparation, representing sample analysis directly from the fermentation vessel. Sampling was done in triplicate for each treatment. A total of 81 samples were collected during the experiment.

2.3. *Spectrophotometric analysis*. All reference data analysis was performed with UV-Vis spectroscopy using a Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The methodology reported by Iland, P., Ewart, A., Sitters, J., Markides, A., & Bruer (2000) was used to quantify total phenolics (IU) and total anthocyanins (mg/L). One hundred µl of sample supernatant was diluted 50 times with 1 M hydrochloric acid, vortexed and stored in the dark for 1 hour before recording the absorbance at 280 nm and 520 nm, respectively. Total phenolic content was calculated as the absorbance at 280 nm multiplied by the dilution factor while total anthocyanins was calculated in malvidin-3-glucoside equivalents using the absorbance at 520 nm.

137 The methyl cellulose precipitable tannin assay (MCP) protocol modified by Mercurio et al.
138 (2007) was used to calculate the total condensed tannins. The tannin content is calculated
139 using the difference between control and treated samples and converted into epicatechin
140 equivalents (mg/L) using a calibration curve and a dilution factor of 40. The 2 ml microfuge
141 treatment tubes consist of adding 600 µl of MCP solution (0.04% w/v) to 50 µl of wine. After
142 being vortexed and standing for 2-3 min, 400 µl of ammonium sulphate and 950 µl of distilled
143 water are added. The control tubes contain no MCP solution and therefore a total of 1.55 ml
144 distilled water is added. Both control and treatment stand for 10 min before being centrifuged
145 at 10 000 rpm for 5 min and recording the absorbance at 280 nm.

146 Colour density was calculated as the sum of absorbance at 420 nm, 520 nm and 620 nm
147 wavelengths for a 50 µl sample volume (Glories, 1984). Polymeric pigments were calculated
148 using the modified Somers assay whereby 200 µl of sample supernatant is diluted with 1.8 ml
149 buffer solution (12% v/v ethanol, 0.5 g/L w/v tartaric acid at pH 3.4) containing 2.5 % sodium
150 metabisulfite (Mercurio et al., 2007). The samples were stored for an hour before calculating
151 the polymeric pigment content in absorption units (AU) using a dilution factor of 10 and the
152 absorbance at 520 nm.

153 ***2.4.*** *Fluorescence analysis*. Front-face fluorescence analysis was conducted at room
154 temperature in a 700 µl quartz cuvette (2 mm width) (Hellma Analytics, Germany) using a
155 Perkin Elmer LS50B spectrophotometer. Excitation wavelengths between 245 nm and 400 nm
156 at 5 nm intervals were used to capture emission spectra between 245 nm and 500 nm at 0.5
157 nm intervals. A 2 cm in diameter aperture was fitted in the emission path for reducing excess
158 light scattering. A scanning speed of 500 nm/min was used and the excitation and emission

slit widths were set at 3 nm and 5 nm, respectively. The instrument control and data manipulation software, UV Winlab, was used for data acquisition.

2.5. CHEMOMETRICS

2.5.1. Data pre-processing. Unwanted spectral signatures were removed using the method described by Airado-Rodríguez et al. (2011) whereby first and second order Rayleigh scattering are excluded as the excitation peaks centred on the identity bands λex = λem and 2λex = λem, respectively. The triangular region below the identity line λex > λem possesses no chemical information and values were therefore inserted as zero. Data and image processing were performed with JupyterLab (Project Jupyter, USA) using the Python 3 language library scikit-learn (Pedregosa et al., 2011) and Matlab ver 9.5 (The Mathworks Inc., MA, USA).

2.5.2. Model validation. Principal component analysis (PCA) was performed on the dataset to evaluate for differences between sample preparation treatments as well as to determine differences based on the stage of fermentation (early versus late).

Two different strategies were followed to assess the influence of sample conditions, namely clean, degassed and unaltered, on the ability of fluorescence spectral properties to accurately predict phenolic content during the fermentation process. First (strategy 1), the regression models reported in dos Santos et al. (2022) and built with clean samples (degassed by vacuum followed by centrifugation) and a more extensive sample set were validated using the 81 samples obtained from the abovementioned Cabernet Sauvignon fermentations. In other words, the models were used to predict the samples obtained from the fermentations performed in this study. Models were built for five phenolic parameters, namely total phenolics (IU), total condensed tannins (mg/L), total anthocyanins (mg/L), colour density (AU) and polymeric pigments (AU) and showed R^2 and validation errors (RMSEP) of 0.77 and 7.16

IU, 0.80 and 172.37 mg/L, 0.77 and 76.57 mg/L, 0.64 and 3.10 AU, 0.66 and 0.49 AU, respectively for the abovementioned phenolic measurements (dos Santos et al., 2022). Overall, models built using a well-balanced dataset and large number of both fermenting musts and finished wines may be generally better suited for all applications compared to those built for specific tasks, which may become over-fitted and predict poorly on new data. Additionally, models built using a more variable dataset may be able to handle the complexity from complex environments such as with degassed or unaltered samples. In addition, the data was passed into each phenolic model to determine the prediction accuracy for different dataset configurations. These sub datasets investigated day of fermentation (all treatment samples for the entire fermentation, day 1-3 treatment samples and day 5-12 treatment samples) and subsequently the three treatments (clean (A), degassed (B) and unaltered (C)). The metrics used to determine prediction accuracy included root mean square error (RMSE) and mean absolute error (MAE). MAE weights all errors equally while RMSE gives errors with larger absolute values more weight than errors with smaller absolute values. Both metrics are regularly used in model evaluation and there is often little consensus when deciding on the most suitable metric, therefore the combination of both allows for improved understanding of different data projections and characteristics of model performance (Chai & Draxler, 2014).

Secondly (strategy 2), calibration models built directly with fluorescence spectra collected from samples in different formats (clean, degassed and unaltered) were attempted. For this, the fermentation data was separately passed through the machine learning pipeline and modelled using the previously optimised parameters identified per phenolic model as reported in dos Santos et al. (2022). Briefly, the fermentation data was split into train and test sub datasets, of which 10 samples were retained as the test validation set. Thereafter, the training data was passed through the five consecutive steps of the pipeline including a column

selector for optimised spectral region selection, a savgol transform used to apply a Savitzky-Golay filter for data smoothing (Savitzky & Golay, 1964), a pre-processing selector for optimal data scaling, six-component PCA for data decomposition, and lastly, the XGBoost regressor to build a tree-based gradient boosted model (Chen & Guestrin, 2016). The total phenolics model consisted of region selection between 260-360 nm excitation and 370-400 nm emission, the total condensed tannins model made use of region selection between 285-340 nm excitation and 290-350 nm emission and the total anthocyanins model involved region selection between 280-300 nm excitation and 330-380 nm emission, all of which were previously identified as optimal spectral regions by dos Santos et al. (2022). Bayesian optimisation was the framework used for the automatic tuning of the other pipeline hyper-parameters such as data scaling and smoothing (Pelikan et al., 2006; Swersky & Adams, 2013). Once passed through the pipeline, 2-fold cross-validation was performed, with the reported RMSE used as the key metric for Bayesian optimisation and the sequential improvement on previously chosen hyper-parameters. The best final model was evaluated using the previously retained 10 sample test set as a form of external validation. The metrics used to determine prediction accuracy included coefficient of determination (R^2cal and R^2val), root mean square error (RMSE) and mean absolute error (MAE). Data processing was performed with JupyterLab (Project Jupyter, USA) using the Python 3 language library scikit-learn (Pedregosa et al., 2011).

3. RESULTS AND DISCUSSION

3.1. *Principal component analysis (PCA).* PCA was conducted on the excitation-emission matrices (EEMs) of the samples collected throughout fermentation. Figure 1 shows the evolution of fluorescence within the fermenting must as the fermentation proceeds, with early fermenting samples (days 1-3) being clustered separately to those of later fermenting

231 samples (days 5-12). Components 1 and 2 accounted for 85.82 % and 7.76 % of the explained
232 variance, respectively. This confirms previous findings involving the difference in fluorescence
233 between fermenting musts and wine, while further highlighting the unique fluorescent
234 changes taking place within a fermentation vessel (dos Santos, 2021). Without having
235 fluorescent information for day 4 of the fermentation, the exact moment in which the
236 fluorescence evolves from characteristically being early versus later in fermentation is
237 unknown. The clear separation between classes, however, may indicate a threshold,
238 potentially the result of maximum plateaued anthocyanin extraction and the subsequent
239 reabsorption of light from a darker sample matrix reached early on in fermentation (Casassa
240 & Harbertson, 2014). Figure 2 shows PCA based on sample preparation treatment. No clear
241 distinction between the treatments is found and may indicate that the stage of fermentation
242 has a greater effect on the fluorescent information obtained than sample preparation.

243 It is important to mention that the freezing of the grape berries could have led to an enhanced
244 presence of solid particles due to the disruption of the berry cells. This could have simulated
245 riper berries with structurally weaker cells (Garrido-Bañuelos et al., 2022) or most of all
246 created suitable conditions to investigate the influence of augmented presence of solid
247 particles on the fluorescence spectral properties of samples during fermentation and on the
248 prediction accuracy of the attempted models. Considering the above, Figure 2 can be seen as
249 a visual representation of the features of front-face fluorescence spectroscopy, whereby the
250 changed sample geometry allows for the analysis of samples in their natural state in order to
251 retain the influence of the surrounding matrix on highly sensitive fluorophores (Airado-
252 Rodríguez et al., 2011; Karoui & Blecker, 2011). The scattered appearance of the samples
253 analysed in triplicate may indicate the heightened sensitivity of fluorescence spectroscopy
254 (Strasburg & Ludescher, 1995). Although all samples were analysed at room temperature and

pipetted into the cuvette as homogenous solutions, other influencing factors must be considered such as the varying rates at which the turbidity settles out in the cuvette over a 25 minute analysis time, the occurrence of which may mimic the turbidity changes occurring naturally during fermentation, as well as the time taken for the analysis of all samples thereby influencing potential instrumental drift or changes in lamp intensity and heating (Airado-Rodŕiguez et al., 2009; Andersen & Bro, 2003).

3.2. *Fermentation excitation-emission matrices*. The three-dimensional EEMs of treatments A, B and C on the first and last day of fermentation are shown in Figure 3. The fluorescent intensity of the clean sample on day 1 is greater than those of the degassed and unaltered samples which may be attributed to the reduction in fluorescence because of turbidity, however, the effect on scattered light is increased for the turbid samples as can be seen in the elevated spectra alongside the removed identity bands λex = λem and 2λex = λem of first and second order Rayleigh scattering, respectively. As fermentation is completed, all treatments experience a reduced fluorescence intensity with treatment A decreasing by roughly 300 units and treatments B and C by roughly 200 units.

This may be a result of the greater fluorescent abilities of monomeric pigments compared to their polymerised counterparts as suggested in previous studies (dos Santos et al., 2022) as well as colour changes occurring by means of anthocyanin extraction. Darker samples are known to reduce fluorescence intensity due to their increased reabsorption of light (Karoui & Blecker, 2011).

Treatments B and C show no major differences between each other and the effect of carbon dioxide (CO_2) during fermentation may not substantially influence fluorescence spectra. The EEMs of treatments B and C in Figure 3 indicate a shouldered peak compared to treatment A,

roughly determined as the region 275-295 nm excitation and 320-360 nm emission (Region 1). Treatment A indicates a slightly more prominent fluorescence determined between 255-265 nm excitation and 360-400 nm emission (Region 2). As fermentation proceeds, these regions become more pronounced specifically with region 2 fluorescing more intensely between 320 and 340 nm emission.

The fluorescence in region 1 correlates well with the regions identified as flavan-3-ols, namely catechin, epicatechin and epigallocatechin, as well as polymeric proanthocyanidins (Airado-Rodríguez et al., 2011; Ranaweera et al., 2021) and may represent the extraction of condensed tannins during fermentation and their subsequent polymerisation. Region 2 falls within the optimal region previously selected by a machine learning pipeline for a total phenolics model (dos Santos et al., 2022) and will be elaborated on below. Additionally, treatments B and C have slightly greater fluorescent intensities at the end of fermentation which may be a result of light scattering.

3.3. *Strategy 1: Model validation*. The best models per phenolic parameter obtained in dos Santos et al. (2022) were validated using the 81 samples collected throughout fermentation. This model validation involved obtaining the prediction accuracy, by means of root mean square error (RMSE) and mean absolute error (MAE), for various sub datasets. Each phenolic model has its own unique set of parameters, with the column selector having identified optimal spectral regions for total phenolics (excitation 260-360 nm and emission 370-400 nm), total condensed tannins (excitation 285-340 nm and emission 290-350 nm) and total anthocyanins (excitation 280-300 nm and emission 330-380 nm). The colour density and polymeric pigments models cover the entire EEM obtained during fluorescence analysis. During model development, ten-fold cross validation was incorporated to prevent over-fitting

and better understand model stability and performance while internally validating the model. The use of this external validation set of fermenting samples aids in investigating the suitability of the chosen parameters per phenolic model, explores model performance when predicting on unseen data and investigates the influence of sample preparation on prediction accuracy. Due to the differences in fluorescence according to the day of fermentation, three sub datasets were explored including the entire fermentation from day 1 to 12, early fermentation from day 1 to 3 and later fermentation from day 5 to 12. Although PCA did not clearly distinguish between sample preparation treatments, these were included as sub datasets to determine model performance under fermentation conditions, including potential effects from CO_2 and turbidity. The spectrophotometric reference data per phenolic parameter is reported in Table 1 below.

Figure 4a shows the performance of the total phenols model possessing a calibration RMSE and MAE of 5.71 index values. The model performed better during early fermentation and generally had the greatest prediction accuracy with degassed and unaltered samples (treatments B and C) with the lowest overall RMSE and MAE for treatment C. On average, the model was able to predict the validation samples within 9.55 and 8.68 index units for RMSE and MAE, respectively. The optimal total phenolic region identified during model development slightly overlaps region 255-265 nm excitation and 360-400 nm emission (Region 2) as identified in Figure 3 and could potentially be influencing the model's prediction abilities. Unaltered samples seemed to have more intense fluorescence in this region and perhaps building the model on clean samples allowed for over-fitting on regional spectral properties and was therefore able to better predict on samples with greater turbidity. Only slightly higher errors were observed for the degassed (B) and unaltered (C) treatments when all the samples were pooled together, indicating minimal model depreciation to predict

samples with CO_2 or solids in suspension. Similar attempts to quantify total phenol content with spectroscopy applications have been reported in the literature (Lambrecht et al., 2022a, 2022b). These studies investigated calibrations using infrared instruments suitable for direct online measurements and using samples with different pre-treatments such as degassing, rough filtration or even no sample pre-treatment. Error values in the range of the calibration model but lower than our validation errors were reported. However, these results need to be contextualized since in this study models built with clean samples are used to predict samples that have received degassing, or no pre-treatment (unaltered) and a certain model depreciation should be expected.

The total condensed tannins model performance is seen in Figure 4b with no clear effect based on the day of fermentation but rather predicting better on clean samples (treatment A). On average, the model was able to predict the validation samples within 196.41 and 172.43 mg/L when compared with the calibration model's RMSE and MAE of 104.03 mg/L. When looking at region 2 (255-265 nm excitation and 360-400 nm emission) identified in Figure 3, the inverse effect of region 1 and total phenols may be occurring, with potential spectral interferences caused by the turbidity of samples reducing the prediction accuracy. Gilmore et al. (2022) reported calibrations for polymeric tannins quantified with HPLC with fluorescence data from diluted grape phenolic extracts. Another study showed fluorescence calibration for MCP total tannin using gradient boost regression in finished Cabernet Sauvignon samples (Schober et al., 2022). Accurate prediction errors were reported in both studies confirming the suitability of fluorescence spectroscopy to quantify tannin content.

The total anthocyanins model was able to predict the fermenting samples on average within 123.13 and 114.21 mg/L when looking at RMSE and MAE, respectively. The model seemed to

perform best during early fermentation (days 1 to 3) and on clean samples as seen in Figure 4c. The optimal spectral region identified during model development slightly overlaps region 2 identified in Figure 3 and may be influenced by the shouldered peak of the turbid samples as described for total condensed tannins. In a study reported by Lambrecht et al. (2022a) partial least square regression (PLS) models for anthocyanin content were attempted for unaltered wine fermenting samples using mid infrared spectra. In agreement with our study, certain permissible model depreciation was also observed due to the presence of particles in suspension. Other studies using fluorescence properties in diluted wine and grape phenolic extracts also reported accurate models to quantify the content of anthocyanins (Schober et al., 2022; Gilmore et al., 2022)

The colour density model performed most poorly of all the models, showing no clear preference for day of fermentation or sample preparation and on average predicting within 7.419 and 6.810 AU compared to the calibration model's RMSE of 2.46 AU (Figure 4d). This may be a result of an optimistically cross-validated model as well as the metric of colour density itself. Colour density is an estimation of responsible yellow, red and blue colouring pigments at three UV-Visible spectral regions (Glories, 1984) and therefore the reflexion of these into the fluorescence EEM may not have been adequately achieved during model development (dos Santos et al., 2022). Contrarily to what was observed here, accurate models to quantify colour density making use of mid infrared spectra have been reported (Lambrecht et la., 2022a)

The polymeric pigments model performed the best, on average predicting within 0.371 and 0.307 AU when compared to the calibration model's RMSE of 0.63 AU. The best model performance can be seen in Figure 4e during later fermentation (days 5 to 12) and for

371 degassed and unaltered samples (treatments B and C). This improved prediction accuracy of
372 the external validation set may be a result of cultivar specific benefits or the polymeric
373 pigments range developing throughout fermentation falling within a region of the calibration
374 model better able to predict. Although possessing seemingly poorer accuracy metrics
375 reported in dos Santos et al. (2022), the model as seen in Figure 5 shows a relatively accurate
376 prediction ability when analysing samples below 2 AU and incorporating more samples within
377 the minority group above this threshold may improve upon the model's predictive ability. This
378 illustrates the importance of balanced datasets in modelling.

379 Prediction models are known to perform better on data used to construct them than new and
380 unseen data, resulting in some expected model depreciation during validation. However,
381 internal validation techniques such as cross-validation or bootstrapping are often
382 optimistically accepted without validating on external data (Bleeker et al., 2003). Within the
383 five best phenolic models previously developed and herein validated, an important
384 consideration includes the variability and balance of the dataset used for model calibration.
385 Certain regions within the models may predict better than others as can be seen with
386 polymeric pigments and although a synthetic dataset was created during model development
387 in dos Santos et al. (2022) to offset any data imbalances using a synthetic minority over-
388 sampling technique for regression (SMOTER), gaps may still remain and have implications for
389 prediction accuracy. The results of this external validation also follow a single fermentation
390 of a single cultivar and should therefore be further investigated to determine the prediction
391 accuracy on other cultivars and fermentations.

392 ***3.4.*** Strategy 2: *Influence of sample preparation to quantify phenolic content*. The
393 fermenting samples were passed through the machine learning pipeline in order to validate

394 the stability and suitability of the model parameters chosen during model development while
395 most importantly determining the influence of sample preparation and the implications for
396 real-time analysis during fermentation. All models were passed through the same pipeline
397 steps as in dos Santos et al. (2022), excluding the SMOTER algorithm due to sampling taking
398 place in triplicate and thereby creating an already well-balanced dataset, therefore
399 eliminating the need for synthetic samples. The total anthocyanins and polymeric pigments
400 models required the removal of outliers, specifically samples A1 and B3 and samples B4-B6,
401 respectively. As no other phenolic model possessed outliers from the second day of analysis,
402 it is difficult to identify the cause of such significant difference.

403 Table 2 below shows the predication accuracy metrics obtained per phenolic parameter
404 model throughout fermentation for each sample preparation treatment. The fact that
405 samples from a single cultivar were collected on consecutive fermentation days might explain
406 the accuracy of the models obtained dos Santos et al. (2022). Moreover, an important
407 consideration is that the effect of sample preparation may not noticeably influence front-face
408 fluorescence spectroscopy, confirming the findings of Figure 3 above. No clear differences can
409 be identified between treatments, except for colour density and polymeric pigments models.
410 For colour density treatment A may have produced slightly better results. However, the
411 prediction accuracy metrics allows for a more holistic evaluation of model performance as in
412 the case of treatments B and C of colour density. Although the data is poorly fitted as reported
413 by R^2, the RMSE and MAE values are not noticeably different to treatment A. variable results
414 were observed for the polymeric pigments model with the best performance observed for
415 the model built with unaltered samples.

Overall, the obtained models resulted in high correlations and validate the chosen pipeline parameters as well as highlight the potential for building models using unaltered samples, the benefit of which involves the application in analysing samples directly from fermentation vessels. Attempting cultivar specific models and the use of a samples set collected during the duration of the fermentation where noticeable changes in the fluorescence spectral properties are observed might have provided a valid strategy to ensure model accuracy. In addition, triplicate sampling aided in obtaining a well-balanced dataset and should also be a consideration in further modelling. The colour density model, although producing promising results in Table 2, should be approached with caution as model development and external validation performed the most poorly of all the phenolic parameters models and should therefore be further explored with regards to optimal model parameter selection and development. As previously discussed, the characteristics of colour density as a metric may potentially limit the success of modelling in this study due to the fluorescent EEM not encompassing the responsible regions or fluorescence spectral characteristics having not been adequately identified. In addition, discrepancies between calibration and validation statistics were observed for the polymeric pigment model with degassed samples (treatment B). This can be understood as poor model robustness (Ranaweera et al., 2021) and it is speculated that the model might be suffering from overfitting, being therefore unable to accurately predict new samples.

When comparing the above models with literature, it was found that the total condensed tannins model performed the best and presents itself as a promising alternative to other spectrophotometric analysis methods such as UV-Vis and infrared spectroscopies. For example, UV-Vis models developed by Aleixandre-Tudo et al. (2018a) obtained RMSE scores of 239 and 209 mg/L for calibration and prediction, respectively, and can be compared to the

fluorescence model developed previously with a RMSE of 104.03 mg/L and externally validated above to predict on average within 196.409 mg/L. Infrared calibration models built using Fourier transform near infrared (FT-NIR), attenuated total reflectance mid infrared (ATR-MIR) and Fourier transform infrared (FT-IR) spectroscopies showed the same trend (Aleixandre-Tudo et al., 2018b). Moreover, studies using infrared instrumentation suitable for direct measurements on filtered and unaltered samples showed similar prediction errors than those reported here. Specifically, prediction errors between 85.23 and 100.37 mg/L were reported (Lambrecht et al., 2022b). Regarding fluorescence spectroscopy applications, similar model statistics were reported in the literature for Cabernet Sauvignon wines and grape phenolic extracts from multiple varieties (Schober et al., 2022; Gilmore et al., 2022). However, these studies differ from the direct measurement approach reported here in the sample dilution step required before spectra data acquisition. The total condensed tannins model built using unaltered samples possessing R^2cal 0.86, R^2val 0.94, and RMSEC 48.42 mg/L (Table 2) is also able to compete while showcasing the potential for building models using fermenting samples analysed directly from the tank, eliminating the need for sample preparation. Producing similar competitive results, the total phenols and total anthocyanins models built in this study show promise and may too present themselves as successful alternatives in agreement with previous literature (Aleixandre-Tudo et al., 2018a; Aleixandre-Tudo et al., 2018b, Lambrecht et al., 2022a, 2022b). When looking at fluorescence spectroscopy in literature, models have previously been built on pure compounds such as catechin and epicatechin (Airado-Rodŕiguez et al., 2009; Cabrera-Bañegil et al., 2017) and also for individual and total content of the main phenolic compounds (Ranaweera et al., 2021; Schober et al., 2022; Gilmore et al., 2022). However, these investigations did not follow a direct measurement approach as the one reported in the present study. Raman spectroscopy

464 calibrations for Cabernet Sauvignon wine phenolics have been successfully investigated and
465 although based on competing phenomena, the fermentation models described in Table 2 can
466 be considered comparatively successful in their prediction accuracies (Gallego et al., 2011).

467 **4. CONCLUSION**

468 Monitoring phenolic content during winemaking may aid in the decision making and
469 implementation of vinification practices thereby improving process control and fermentation
470 management. This study validated the potential for phenolic models built using fluorescence
471 spectroscopy and chemometrics as well as the suitability of front-face geometry to quantify
472 phenolics of fermenting musts under fermentation conditions. Following a Cabernet
473 Sauvignon fermentation allowed for improved understanding of the evolution of fluorescence
474 spectra from juice to wine. The models were adequately validated and show the potential for
475 analysing directly from the fermentation vessel which may allow for phenolic analysis using
476 portable optical devices or on-line automated systems. The potential for building
477 fermentation-based models appears promising and may be beneficial to winemakers in
478 creating cellar specific software able to be expanded on each vintage and used as a tool for
479 optimal red wine production.

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483 **REFERENCES**

484 Airado-Rodríguez, D., Durán-Merás, I., Galeano-Díaz, T., & Wold, J. P. (2011). Front-face
485 fluorescence spectroscopy: A new tool for control in the wine industry. *Journal of Food*
486 *Composition and Analysis*, *24*(2), 257–264. https://doi.org/10.1016/j.jfca.2010.10.005

Airado-Rodŕiguez, D., Galeano-Díaz, T., Durán-Merás, I., & Wold, J. P. (2009). Usefulness of fluorescence excitation-emission matrices in combination with parafac, as fingerprints of red wines. *Journal of Agricultural and Food Chemistry*, *57*(5), 1711–1720. https://doi.org/10.1021/jf8033623

Aleixandre-Tudo, J. L., Buica, A., Nieuwoudt, H., Aleixandre, J. L., & du Toit, W. (2017). Spectrophotometric analysis of phenolic compounds in grapes and wines. *Journal of Agricultural and Food Chemistry*, 65(20), 4009-4026. https://doi.org/10.1021/acs.jafc.7b01724

Aleixandre-Tudo, J.L., Nieuwoudt, H., Aleixandre, J. L., & du Toit, W. (2018). Chemometric compositional analysis of phenolic compounds in fermenting samples and wines using different infrared spectroscopy techniques. *Talanta*, *176*(May 2017), 526–536. https://doi.org/10.1016/j.talanta.2017.08.065

Aleixandre-Tudo, Jose Luis, Nieuwoudt, H., Aleixandre, J. L., & du Toit, W. (2018). Chemometric compositional analysis of phenolic compounds in fermenting samples and wines using different infrared spectroscopy techniques. *Talanta*, *176*, 526–536. https://doi.org/10.1016/j.talanta.2017.08.065

Aleixandre-Tudo, Jose Luis, Nieuwoudt, H., Olivieri, A., Aleixandre, J. L., & du Toit, W. (2018). Phenolic profiling of grapes, fermenting samples and wines using UV-Visible spectroscopy with chemometrics. *Food Control*, *85*, 11–22. https://doi.org/10.1016/j.foodcont.2017.09.014

Andersen, C. M., & Bro, R. (2003). Practical aspects of PARAFAC modeling of fluorescence excitation-emission data. *Journal of Chemometrics*, *17*(4), 200–215. https://doi.org/10.1002/cem.790

510 Bleeker, S. E., Moll, H. A., Steyerberg, E. W., Donders, A. R. T., Derksen-Lubsen, G., Grobbee,
511 D. E., & Moons, K. G. M. (2003). External validation is necessary in prediction research:
512 A clinical example. *Journal of Clinical Epidemiology*, *56*(9), 826–832.
513 https://doi.org/10.1016/S0895-4356(03)00207-5

514 Cabrera-Bañegil, M., Hurtado-Sánchez, M. del C., Galeano-Díaz, T., & Durán-Merás, I. (2017).
515 Front-face fluorescence spectroscopy combined with second-order multivariate
516 algorithms for the quantification of polyphenols in red wine samples. *Food Chemistry*,
517 *220*, 168–176. https://doi.org/10.1016/j.foodchem.2016.09.152

518 Cadot, Y., Miñana-Castelló, M. T., & Chevalier, M. (2006). Anatomical, histological, and
519 histochemical changes in grape seeds from Vitis vinifera L. cv Cabernet franc during fruit
520 development. *Journal of Agricultural and Food Chemistry*, *54*(24), 9206–9215.
521 https://doi.org/10.1021/jf061326f

522 Canals, R., Llaudy, M. C., Valls, J., Canals, J. M., & Zamora, F. (2005). Influence of ethanol
523 concentration on the extraction of color and phenolic compounds from the skin and
524 seeds of tempranillo grapes at different stages of ripening. *Journal of Agricultural and*
525 *Food Chemistry*, *53*(10), 4019–4025. https://doi.org/10.1021/jf047872v

526 Casassa, F. L., & Harbertson, J. F. (2014). Extraction, Evolution, and Sensory Impact of Phenolic
527 Compounds During Red Wine Maceration. *Annual Review of Food Science and*
528 *Technology*, *5*(1), 83–109. https://doi.org/10.1146/annurev-food-030713-092438

529 Chai, T., & Draxler, R. R. (2014). Root mean square error (RMSE) or mean absolute error
530 (MAE)? -Arguments against avoiding RMSE in the literature. *Geoscientific Model*
531 *Development*, *7*(3), 1247–1250. https://doi.org/10.5194/gmd-7-1247-2014

532 Chen, T., & Guestrin, C. (2016). XGBoost: A scalable tree boosting system. *Proceedings of the*

ACM SIGKDD International Conference on Knowledge Discovery and Data Mining, *13-17-Augu*, 785–794. https://doi.org/10.1145/2939672.2939785

Dos Santos, I. A. (2021). The quantification of red wine phenolics using fluorescence spectroscopy with chemometrics (*Master's thesis dissertation*, Stellenbosch University, Stellensboch, South Africa).

dos Santos, I., Bosman, G., Aleixandre-Tudo, J. L., & du Toit, W. (2022). Direct quantification of red wine phenolics using fluorescence spectroscopy with chemometrics. *Talanta*, *236*(September 2021), 122857. https://doi.org/10.1016/j.talanta.2021.122857

Gallego, Á. L., Guesalaga, A. R., Bordeu, E., & González, Á. S. (2011). Rapid measurement of phenolics compounds in red wine using raman spectroscopy. *IEEE Transactions on Instrumentation and Measurement*, *60*(2), 507–512. https://doi.org/10.1109/TIM.2010.2051611

Garrido-Banuelos, G., Buica, A., & du Toit, W. (2022). Relationship between anthocyanins, proanthocyanidins, and cell wall polysaccharides in grapes and red wines. A current state-of-art review. *Critical reviews in food science and nutrition*, 62(28), 7743-7759. https://doi.org/10.1080/10408398.2021.1918056

Gilmore, A. M., Sui, Q., Blair, B., & Pan, B. S. (2022). Accurate varietal classification and quantification of key quality compounds of grape extracts using the absorbance-transmittance fluorescence excitation emission matrix (A-TEEM) method and machine learning. *OENO One*, 56(4), 107-115. DOI: https://doi.org/10.20870/oeno-one.2022.56.4.5561

Glories, Y. (1984). La couleur des vins rouges, 2eme partie. *Connaissance de La Vigne et Du Vin*, *18*, 253–271.

556 Iland, P., Ewart, A., Sitters, J., Markides, A., & Bruer, N. (2000). *Techniques for chemical*
557 *analysis and quality monitoring during winemaking*. Patrick Iland Wine Promotions.

558 Karoui, R., & Blecker, C. (2011). Fluorescence Spectroscopy Measurement for Quality
559 Assessment of Food Systems-a Review. *Food and Bioprocess Technology*, *4*(3), 364–386.
560 https://doi.org/10.1007/s11947-010-0370-0

561 Lambrecht, K., Nieuwoudt, H., du Toit, W., & Aleixandre-Tudo, J. L. (2022a). Moving towards
562 in-line monitoring of phenolic extraction during red wine fermentations using infra-red
563 spectroscopy technology. Influence of sample preparation and instrumentation. *Journal*
564 *of Food Composition and Analysis*, 110, 104542.
565 https://doi.org/10.1016/j.jfca.2022.104542

566 Lambrecht, K., Nieuwoudt, H., Du Toit, W., & Aleixandre-Tudo, J. L. (2022). Optimisation of
567 PLS Calibrations for Filtered and Untreated Samples towards In-Line Monitoring of
568 Phenolic Extraction during Red-Wine Fermentations. *Fermentation*, 8(5), 231.
569 https://doi.org/10.3390/fermentation8050231

570 Mercurio, M. D., Dambergs, R. G., Herderich, M. J., & Smith, P. A. (2007). High throughput
571 analysis of red wine and grape phenolics - Adaptation and validation of methyl cellulose
572 precipitable tannin assay and modified somers color assay to a rapid 96 well plate
573 format. *Journal of Agricultural and Food Chemistry*, *55*(12), 4651–4657.
574 https://doi.org/10.1021/jf063674n

575 Pedregosa, F., Varoquaux, G., Gramfort, A., Michel, V., Thirion, B., Grisel, O., Blondel, M.,
576 Prettenhofer, P., Weiss, R., Dubourg, V., Vanderplas, J., Passos, A., Cournapeau, D.,
577 Brucher, M., Perrot, M.and Duchesnav, E. (2011). *Machine Learning in Python*. *12*, 128–
578 154. https://doi.org/10.4018/978-1-5225-9902-9.ch008

Pelikan, M., Goldberg, D. E., & Tsutsui, S. (2006). Hierarchical Bayesian optimization algorithm: toward a new generation of evolutionary algorithms. *Choice Reviews Online*, *43*(05), 43-2847-43–2847. https://doi.org/10.5860/choice.43-2847

Sacchi, K. L., Bisson, L. F., & Adams, D. O. (2005). A review of the effect of winemaking techniques on phenolic extraction in red wines. *American Journal of Enology and Viticulture*, *56*(3), 197–206.

Savitzky, A., & Golay, M. J. E. (1964). Smoothing and Differentiation of Data by Simplified Least Squares Procedures. *Analytical Chemistry*, *36*(8), 1627–1639. https://doi.org/10.1021/ac60214a047

Schober, D., Gilmore, A., Chen, L., Zincker, J., & Gonzalez, A. (2022). Determination of Cabernet Sauvignon wine quality parameters in Chile by Absorbance-Transmission and fluorescence Excitation Emission Matrix (A-TEEM) spectroscopy. *Food Chemistry*, 392, 133101. https://doi.org/10.1016/j.foodchem.2022.133101

Smith, P. A., Mcrae, J. M., & Bindon, K. A. (2015). Impact of winemaking practices on the concentration and composition of tannins in red wine. *Australian Journal of Grape and Wine Research*, *21*, 601–614. https://doi.org/10.1111/ajgw.12188

Strasburg, G. M., & Ludescher, R. D. (1995). Theory and applications of fluorescence spectroscopy in food research. *Trends in Food Science & Technology*, *6*(3), 69–75. https://doi.org/10.1016/S0924-2244(00)88966-9

Swersky, K., Snoek, J. & Adams, R. P. (2013). Multi-task Bayesian optimization. *Advances in Neural Information Processing Systems*, 26, 2004-2012.

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