The effect of wine age and tannin composition on tannin activity across consecutive Pinotage red wine vintages

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ABSTRACT

The effect of wine age on tannin chemistry and tannin activity values was investigated in red wine extracts according to a chemosensory approach. Sixteen Pinotage red wines of multiple vintages (2003-2018) were each isolated into four distinct polymeric fractions (F1-F4) on Sephadex LH-20 by flash-column chromatography. Tannin activity increased with molecular weight, where F1 was associated with positive values, reported for the first time, suggesting a different interaction mechanism than the usual exothermic value obtained in more polymerised samples such as F2 and F3. Weakly positive correlations were observed between tannin activity and tannin concentration by RPLC. Moderately positive correlations were obtained between either tannin estimation techniques, yet this was highly dependent on wine age and molecular size. Overall, tannin activity decreased across all vintages as a function of wine age, indicative of age-related reactions such as tannin oxidation, cleavage and precipitation. Chemosensory studies showed that while activity values across all fractions explained little variation, lowly positive correlations existed between larger polymeric fractions (F3 and F4) and mouthfeel attributes, namely astringency and bitterness, suggesting that highly polymerised tannins with higher activity values play a significant role in mouthfeel modification. More research is required to understand the actual sensory perception of proanthocyanidin size in red wine proanthocyanidin extracts and tannin-derived pigments (as opposed to wine) and to what extent it is influenced by parameters such as tannin activity across long-term ageing.

KEYWORDS: Sephadex LH-20, tannin activity, polymerisation, principal component analysis, astringency, chemosensory, wine age
INTRODUCTION

From the onset of red winemaking operations, such as fermentation to long-term ageing, there are several chemical reactions which affect the tannin chemistry of red wines. These reactions may directly or indirectly modify the chemical composition and, therefore, also the sensory attributes of red wine.

The chemical reactions between tannins as well as tannins and anthocyanins have been well-documented. The adducts formed in these reactions may be formed in either the absence of oxygen or by acetaldehyde-mediated polymerisation (Cheynier et al., 2006). The reactivity of anthocyanins with tannins also depends on structural factors such as size and composition, both of which are linked to tannin origin. Anthocyanin-tannin reactions play an important role in red wine colour stabilisation through the formation of polymeric pigments, as processes such as micro-oxygenation occur during wine ageing. From a sensory perspective, studies have shown that higher tannin concentrations where the degree of polymerisation is also larger (Soares et al., 2011) may lead to an increase in astringency perception (Cáceres-Mella et al., 2013; Mercurio and Smith, 2008). The incorporation of anthocyanin-tannin adducts (polymeric pigments) into the wine matrix instead has been shown to indirectly reduce the intensity of astringency, as they may hinder the interaction between tannins and proteins (García-Estévez et al., 2018).

Similarly, research by other authors suggests certain anthocyanins, in addition to copigments, may play a role in the puckering sensation and bitterness of red wine (Gawel et al., 2007; Soares et al., 2017).

Reactions involving tannins and proteins arguably play a greater role in affecting red wine mouthfeel properties than anthocyanin-tannin reactions. Regardless, astringency is a tactile, puckering sensation that occurs according to a three-step mechanism mainly governed by non-covalent forces, namely hydrophobic interactions and hydrogen bonding, primarily between condensed tannins and salivary (proline-rich) proteins (Charlton et al., 2002). As red wine ages, tannins or proteins may be subject to other reactions. Firstly, tannins may interact with other macromolecular structures, such as certain polysaccharides, which have been shown to reduce astringency (Carvalho et al., 2006; Poncet-Legrand et al., 2007), possibly since tannins are partially bound to polysaccharides react less strongly with salivary proteins. This may be explained either by the formation of a ternary polysaccharide-tannin-protein complex or by competition between polysaccharides and protein for sites on the tannin molecule (Brandão et al., 2017).

Secondly, salivary proteins are also capable of interacting with either hydrolysable tannins (Soares et al., 2019) or pyranoanthocyanins (García-Estévez et al., 2017), both of which have been shown to modulate red wine mouthfeel attributes such as astringency and bitterness.

As red wine ages, astringency is known to decrease over time. This may be due to several factors, including tannin concentration, although aged and young wines with similar concentrations may exhibit variable degrees of astringency between them (Mercurio and Smith, 2008). This suggests that structural modifications of tannins may affect mouthfeel. These include tannin size as tannins of higher molecular weight, usually more astringent than the low-molecular-weight flavanols, are thought to contain fewer sites for interactions with proteins because of a steric hindrance (Cheynier et al., 2006). The interactions of tannins with proteins and polysaccharides over time may also modulate the perception of astringency (Pascal et al., 2007; Poncet-Legrand et al., 2010), in addition to the abovementioned direct and indirect anthocyanin-tannin or tannin-tannin interactions which form polymeric pigments and tannin polymers, respectively. As proanthocyanidins reach insoluble molecular sizes, they may also precipitate from the wine matrix, leading to a reduction in astringency (Teng et al., 2019; Teng et al., 2021). Cleavage reactions may form smaller tannins, such as monomers, which are perceived as more bitter than astringent (Kennedy and Jones, 2001; Vidal et al., 2004).

Additionally, the formation of flavanol sulphonates—reactions between condensed tannins and SO$_2$—have been shown to reduce astringency (Ma et al., 2018). Finally, matrix effects may also play a role in modulating red wine mouthfeel and include pH, residual sugar and ethanol levels (Fontoin et al., 2008). Therefore, a variety of chemical interactions occur during ageing, which gradually affects the mouthfeel of red wine over time. This also shows that tannin concentration is merely one of the tannin features involved in astringency.

Tannin activity, a thermodynamic measurement explored by multiple authors from 2010 onwards (Barak and Kennedy, 2013; Revelette et al., 2014; Sáenz-Navajas et al., 2019), has been used as a possible analytical measurement of tannin affinity to proteins and, therefore, astringency. Reversed-phase liquid chromatography with diode array detection (RPLC-DAD) has been the preferred technique used to measure tannin activity, as opposed to isothermal titration calorimetry (ITC) (McRae et al., 2010). This technique may be applied to red wines by either direct injection or extracts thereof. Many factors affect tannin activity, including proanthocyanidin subunit size and composition, polymeric pigmentation and tannin oxidation (Yacco et al., 2016), the presence of other macromolecules (Revelette et al., 2014) and SO$_2$ concentrations (Ma et al., 2018). Various authors have used chemosensory approaches to understand how tannin structure, concentration, activity and matrix effects are linked to the sensory properties of wine and its implications for red wine astringency (Ferrero-del-Teso et al., 2020a; Ferrero-del-Teso et al., 2020b; Sáenz-Navajas et al., 2019; Sáenz-Navajas et al., 2018; Sáenz-Navajas et al., 2020; Watrelot et al., 2016).

However, most studies have used data sets where the variation between wines were high (because of cultivar or regional differences). Limited studies have investigated chemosensory studies to evaluate how structure-activity relationships change in a single varietal wine across multiple,
consecutive vintages of a single producer. The main aim of the study is, therefore, to investigate how tannin activity and concentration may be linked to red wine mouthfeel by sensory analysis and how these variables may change according to vintage.

**MATERIALS AND METHODS**

1. **Reagents**

Sephadex LH-20 resin, acetonitrile (99 %, HPLC-grade), acetone (99 %, HPLC-grade), ammonium sulphate, hydrogen chloride (37 %, ACS-reagent), methylcellulose and sodium acetate (anhydrous) were all purchased from Sigma Aldrich. Absolute ethanol (99 %, GC-grade), rectified ethanol (96 %) and trifluoroacetic acid (TFA, spectroscopy-grade, Uvasol®) were purchased from Merck. Formic acid was purchased from Fluka, and α-phosphoric acid (85 %) was purchased from Alfa Aesar (ThermoFisher Scientific). Deionised water was used that of Milli-Q Millipore, while distilled water was also of a similar grade. For HPLC, solvents and solutions were HPLC-grade, and only deionised water was used.

2. **Red wine samples**

Sixteen commercial wine samples (cv. Pinotage)—bottled using cork closures—were used from a well-recognised wine estate in Stellenbosch between the vintage years of 2003 and 2018. Five hundred mL of wine were vacuum-filtered using Munktell filter disks (grade 3 hw) prior to its application to Sephadex LH-20. The wine codes used for each wine sample included the relevant vintage year (2003–2018, shortened to 03–18) and four corresponding fractions (F1: first eluting, F2: second eluting, F3: third eluting and F4: a bulk fraction containing equal aliquots of F1–F3). As an example, 17F2 refers to the second fraction of Pinotage 2017 vintage.

3. **Tannin isolation and purification**

Proanthocyanidins were fractionated and isolated from red wine according to a previous method (Taylor et al., 2003) with slight modifications. Briefly, two Sephadex LH-20 columns of similar dimensions (415 mm L × 27 mm I.D., bed volume ≈ 250 mL) were equipped with a 500 mL solvent reservoir and prepared using a 500 mm glass column equilibrated with two column volumes of 50 % methanol, each containing 0.05 % TFA. Following wine sample loading, both columns were washed with eight sequential 250 mL elution volumes of aqueous methanol and acetone-based solutions, each acidified with 0.05 % TFA, by flash column chromatography (FCC) (pressure: 30–60 kpa; flow rate: 3.5–4 mL/min. Full information, is provided in Table S1). Sephadex columns were connected to a single compressed air line; therefore, the flow rate was split. Frit sizes of the glass columns varied, which resulted in differences in column flow rate.

During FCC, dry air (Afrox, Johannesburg, South Africa) was used to increase the flow rate at which solvent washings and, therefore, solutes were eluted. The different solutions were used to elute proanthocyanidin fractions in terms of increasing molecular mass, as seen previously (Barak and Kennedy, 2013; Taylor et al., 2003). Earlier washings were used to elute both low molecular weight impurities and phenolic material such as low molecular weight monomers and dimers. Contrastingly, later eluents were used for high molecular weight proanthocyanidin oligo- and polymers. Twenty mL fractions were collected in glass vials, and after approximately 100 vials were collected, the column was re-equilibrated with two to three column volumes of 50 % methanol. Fractions of the 50 and 60 % methanol washings [approximate column chromatography (ccf), 1–25] were not analysed for tannin activity, as they contained both low molecular weight non-phenolic impurities and phenolic compounds, including anthocyanins and tannin-derived monomers and dimers. Fractions were stored at 15 °C prior to rotary evaporation. Five fractions were sequentially pooled together, concentrated under reduced pressure at 40 °C to remove the organic solvent, and stored in a -80 °C refrigerator for 12-24 hours. The remaining aqueous portion was lyophilised to a dry powder and stored at -4 °C. The approximate combined mass of the fractions across wine samples—excluding the first 25 column chromatography fractions—was between the range of 2-3 grams of lyophilised extract. The 16 purified wine samples each contained between 16-18 polymeric fractions, which were pooled and grouped into four purified fractions according to column chromatography fraction: F1 (ccf26–35), F2 (ccf36–50) and F3 (ccf51–100, if applicable). This means that for each wine, the total amount of vials collected for each purified fraction (from F1 to F3) was 10, 15 and approximately 50, respectively. Fewer vials were collected for F1 and F2, as more proanthocyanidin material eluted within these ranges. Conversely, more vials were pooled and purified as F3, as the amount of tannin material eluting with this range was considerably less than F2 and F3. The last fraction (F4) is a bulk fraction representing each of the previous three purified fractions within a single sample. Therefore, this fraction contains equal aliquots of either F1 to F3, from ccf 26–100. Therefore, it was thought that F4 is the sample which represents the entire oligo- or polymeric proanthocyanidin fraction of each of the sixteen Pinotage wines. Fractions were grouped in this way to provide variation in tannin structure based on molecular size, which may also reflect differences in total phenolic and tannin content and DP. Overall, 16 wines containing four fractions each gave 64 unique samples whose chemical nature was assumed to be polymeric. All these fractions were used for both spectrophotometric and chromatographic analyses.

4. **Instrumentation**

**HPLC—Tannin activity:** Chromatographic analyses for tannin activity were conducted using components of both the Agilent 1100 and the Hewlett-Packard 1050 series, abbreviated as A and HP, respectively. This system consisted of a model G1379A micro-degasser (A), a G1316B pump (HP), a G1318B autosampler (HP), a G1316A column heater (A), a G1307A DAD/UV-Vis detector (HP) and a 1046A programmable FLD detector (HP). Data analyses were performed using Chemstation for LC 3D systems [Rev. version A 10.02 (1757)].
**HPLC—Phloroglucinolysis**: Chromatographic analyses for phloroglucinolysis were conducted using Agilent hardware. This consisted of a 1260 ALS (G1329B) autosampler, a 1260 binary pump, a 1260 Inf. Micro-degasser, two Chromolith Performance RP-18e columns connected in series, a 1290 (G1330B) thermostat equipped with a 1260 (G1316A) thermostatically controlled compartment (TCC) and a 1260 DAD (G412B). Chemstation software was used to analyse chromatographic data.

**Spectrophotometry**: Spectrophotometry was carried out using a Thermoscientific Multiskan Go Spectrophotometer. Skan RE 5.0 software was used, and where applicable, the k-factor was 0.166.

### 5. Chemical analyses

#### 5.1. Tannin activity

The RPLC-DAD method used to measure tannin activity is primarily based on chromatographic methods by Barak and Kennedy (2013), and Revelette et al. (2014), with slight modifications. Reverse-phase analyses utilised a polystyrene-divinylbenzene (PS-DVB) analytical column (herein referred to as PLRPs instead of PLRP-S, 50 mm × 2.1 mm, 3 μm particle size, 100 Å pore size, Agilent Technologies, South Africa) protected by a guard column containing the same packing material (PLRP-1, 20 mm × 2.1 mm, 3 μm particle size, Hamilton). Briefly, purified wine tannin extracts (5 mg/mL)—isolated using Sephadex LH-20 by FCC—were dissolved in an aqueous buffer solution (consisting of 15 % v/v aqueous methanol, 40 mM sodium acetate, and 20 mM HCl, acidified to a pH of 4.6 with 0.1 M HCl). These tannin isolates were filtered using a 0.22 μm hydrophilic PVDF syringe filter (Stargate Scientific, Wilgeneuvel, South Africa) prior to injection (using a 7 μL injection volume). The following mobile phases were used: A) 1.5 % aqueous phosphoric acid and B) 20 % of A in acetonitrile, with a flow rate of 0.3 mL/min. The linear gradient was performed according to that of Revelette et al. (2014), with slight modifications, allowing an additional 10 minutes of column re-equilibration towards the initial conditions. Therefore, the total time of a chromatographic run was 30 minutes.

To determine tannin activity, samples were run at four column temperatures (in 5 °C increments, from 30-45 °C). A blank sample (the aqueous buffer solution mentioned above) was run at all column temperatures and subtracted from sample signals at the corresponding temperatures to eliminate background interference. All temperatures were converted to kelvin (°K) to calculate tannin activity. Tannin elution was monitored at 280 nm. Following the subtraction of the buffer blank, a baseline was drawn through 0 mAU across the entire sample peak area. Integrations were performed for a given sample at each of the four temperatures, according to Yacco et al. (2016), to obtain a linear, van’t Hoff plot. A value for tannin activity of a given sample was directly calculated from this line by obtaining the value of the slope.

#### 5.2. Tannin concentration

The corresponding tannin concentration of a given sample was determined based on the total tannin peak area, in (−)-epicatechin equivalents (in mg/L), at 30 °C (Yacco et al., 2016). This was based on the same analyses used to obtain tannin activity values. A six-point serial dilution series was generated to a final concentration of 0.31 mg/mL from a 10 mg/mL epicatechin stock solution. Thereafter a calibration curve was used to determine total tannin concentration, measured in mg/L epicatechin equivalents, based on peak area.

#### 5.3. Phloroglucinolysis

The four fractions (F1-F4) of crude extracts obtained from Sephadex LH-20 were analysed by acid-catalysed cleavage in the presence of excess phloroglucinol, using an adaptation of Brillante et al. (2017). Briefly, 5 mg/mL of proanthocyanidin extract in methanol was centrifuged for two minutes and then reacted 1:1 with the phloroglucinol reagent (100 and 20 g/L of phloroglucinol and ascorbic acid in a methanolic solution containing 0.2 N HCl). Samples were allowed to react at 50 °C for 20 minutes, and the cleavage reaction was immediately stopped by adding five volumes of sodium acetate (40 mM). The phloroglucinolysis reaction was duplicated for each individual sample. Following centrifugation for two minutes, samples were capped in vials and analysed by RPLC-DAD at 280 nm. Parameters such as subunit composition, tannin concentration, mDP, mass conversion and galloylation %, and average molecular mass were calculated according to Kennedy and Taylor (2001). The degree of galloylation (in epicatechin gallate) was calculated as the sum of the galloylated extension and terminal subunits expressed as a percentage of the total subunit composition. Theoretical tannin concentrations and conversion yields were calculated (based on catechin, epicatechin and their adducts) according to a previous calibration, in terms of mg/L epicatechin equivalents and % gravimetric recovery, respectively.

#### 5.4. Methylcellulose precipitable (MCP) tannin assay

The total tannin content of the purified tannin isolates was calculated according to the method developed and validated by Sarneckis et al. (2006), with slight modifications. Briefly, 5 mg/mL of tannin extracts were dissolved in a solution of 48 % rectified ethanol (in distilled water), vortexed and sonicated to ensure most of the extract in the solution were fully dissolved. Extracts were centrifuged at 10 000 rpm for two minutes thereafter, prior to MCP analysis. After subjecting samples to the MCP reaction, 200 μL of either control or treatment solution was plated into well-plates and analysed at 280 and 520 nm (30 °C). Tannin concentrations were determined by subtracting absorbance values for a treatment sample from its control [using a blank solution of 48 % aqueous ethanol in distilled water (1:49)] to obtain a corrected absorbance value. This value was plotted on a calibration curve constructed using a seven-point dilution series using distilled water from a 0.1 % epicatechin solution in absolute ethanol. For MCP, all sample measurements were performed in triplicate.
6. Sensory analysis

Sensory analyses were performed within a single day on 15 of the analysed wines (2004-2018), using a panel of 15 wine industry professionals (13 females and two males, all over 25 years of age). Sensory attributes were evaluated using intensity rankings on a discrete scale of 0 to 7, from low to high intensity, respectively. Wines were assigned blinding codes and separated randomly into three sets of tastings/flights to ensure that similar vintage years were not evaluated in quick succession. The red wine samples were sensorially evaluated using non-transparent, black ISO/INAO wine tasting glasses. This was located within the well-ventilated, naturally lit sensory laboratory located at the Department of Viticulture and Oenology, Stellenbosch University. The temperature of the laboratory was kept constant at 20 °C. Panellists were allowed a break of two to three minutes before evaluating a new flight of wines. Compusense Cloud Academic Consortium software was used for sensory data analyses.

7. Multivariate data analyses

All the following multivariate data analyses were generated using Statistica software, Version 14.0.0.15.

7.1. Principle component analysis

Across many disciplines, large datasets are often encountered. Principal component analysis (PCA) is one of many techniques which can be used to reduce data dimensionality and enhance data interpretability while preserving the variability found within a given data set (Jolliffe and Cadima, 2016). In the present study, PCA was used as an exploratory tool to identify both known and potentially unknown correlations among nine chemical variables among four fractions, across 15 red wine vintages (2004-2018), after dimensionality reduction. Only statistically relevant chemical variables were retained and used for PCA.

7.2. Multifactorial analysis

Multifactorial analysis (MFA) is an extension of PCA and integrates different groups of variables describing the same observations, resulting in an integrated picture of observations and relationships between groups of variables (Abdi et al., 2013). For the present study, to gain insight into how chemical and sensory data were related, MFA was performed by integrating the chemical and sensory data (related to astringency and bitterness) on wine vintages 2004-2018.

RESULTS

8. Chemical analyses

The effect of wine vintage on tannin activity, tannin content by either HPLC, MCP and phloroglucinol-based measurements were investigated in red wine extracts isolated from 16 Pinotage wines. Across the 16 wine samples, reference will be made to both older and newer wines, characterised by the vintage years of 2003-2010 and 2011-2018, respectively. All chemical data, including chromatographic and spectrophotometric analyses, are found in Table S2, while all phloroglucinolysis data are available in Tables S3 and S4.

Figure 1 shows how tannin activity values change as a function of both fraction and wine vintage. Positive activity values were observed for a few fractions within F1, with the lowest activity (most positive) reported as 624 J/mol. Tannin activity values were observed to increase across F1 to F3. With F4 constituting equal aliquots of each of the previous polymeric fractions, it was therefore predicted that activity values for F4 would have an intermediate, average value.
per sample between that of F1-F3. This holds true for some wines, but not for all of them. The moving average trendline of F4, depicted as the yellow stippled line in Figure 1, was obtained by using the average value between two data points to generate a point in a line. Generally, these trendlines reduce the fluctuations in normal trendlines to show a smoother pattern. The average trendline illustrates how the bulk, polymeric fraction of the entire wine changes with age. This indicates that activity values do not follow a clear trend across vintages, even within the same wine producer. Additionally, F2 and F3 within older wines had lower activity values than in newer wines.

To gain insight into how tannin content varies according to wine age and fraction, tannin concentrations were determined by RPLC (in terms of mg/L epicatechin equivalents) according to a method on a PLRPs column by Revelette et al. (2014). The data is shown in Table S2 and Figure 2. The results show that tannin concentrations change as a function of both fraction and wine vintage. Overall, tannin concentrations ranged from 1435-5009 mg/L. Besides 12F1, which possessed the lowest tannin concentration, these values increased as a function of wine age within F1, occurring within the range of 2871-5009 mg/L. Within F2, tannin levels varied from 3875-4954 mg/L. No trends were observed within this fraction; in fact, these values appeared to remain consistent during wine ageing. Fraction F3 possessed tannin levels of 1435-4143 mg/L. Generally, tannin concentrations increased as a function of wine age. Finally, the tannin concentrations in F4 (3216-4853 mg/L) generally increased as a function of wine vintage. However, this increase in F4 was not as pronounced as that of F3, given that its concentration difference (1637 mg/L) was smaller than that of both F1 and F3. Holistically, these increases in tannin concentration with wine age in F1, F3 and F4 over time are moderate as not all samples follow this trend (Figure 2). In addition, the tannin content of F4 does not always correspond to the average of fractions F1-F3, similarly to what was observed for the tannin activity data.

Tannin concentrations by MCP were also determined to establish potential structure-activity relationships. MCP tannin concentration ranges fell within the range of 986-6163 mg/L and followed a similar trend within most vintages, an increase from F1 to F2, followed by a decrease from F2 to F3 in all but the 2003, 2006, 2008 and 2015 vintages. In F2, it appears the tannin concentrations are lower in aged wines.

HPLC-phloroglucinolysis was another additional technique used to obtain complementary, quantitative information on wine tannins. Parameters such as tannin concentration, mean degree of polymerisation (mDP), theoretical mass conversion % (MC %), degree of galloylation (expressed in % in the present study) and average molecular weight are all parameters subject to changes as a function of wine age, while they are also known to affect tannin activity.

Phloroglucinol-determined tannin concentrations were determined based on catechin and epicatechin (and their respective phloroglucinol adducts), ranging from 112-975 mg/L epicatechin equivalents. Generally, concentrations across all vintages increased in the order F1, F3 and F2. This is highlighted in the concentration ranges for each fraction, namely F1 (112-670 mg/L), F2 (214-945 mg/L) and F3 (262-872 mg/L). Similarly, average tannin concentrations within each fraction (across all vintages) show a similar trend, increasing in the order F1, F3 and F2 (382, 571 and 503 mg/L). No trends were identified in how tannin concentrations change according to vintages (2003-2018) within a single fraction (i.e., F1-F3), in either

**FIGURE 2.** Tannin concentrations obtained with HPLC-PLRPs per fraction (F1: first eluting fraction; F2: second eluting fraction; F3: third eluting fraction, and F4: bulk fraction) and wine age (vintages 2003-2018).

Considering the various tannin estimation techniques used during the present study, the correlation coefficients between tannin methods have been analysed (Table S5). For older vintages, weak but positive correlations were observed between HPLC-PLRPs and MCP tannins, for F3 and F4, with their r-values equal to 0.38 and 0.4, respectively [Table S5(A)]. However, stronger positive correlations were observed between fractions F1 (0.95) and F3 (0.85), with a moderately positive correlation observed in F4 (0.68); in both cases, these were only applicable to newer vintages. Moderately positive correlations were observed between both assays, within fractions F3 and F4, for all the vintage sets, with their r-values reported as 0.58 and 0.56, respectively. When comparing HPLC methods, poor correlations existed in both age and fraction, with F1 and F3 in young extracts and F2 in aged extracts, giving the highest positive r-values, namely 0.37, 0.48 and 0.38, respectively [Table S5(B)]. Finally, moderately positive correlations for F2 and F3 within all vintages (0.55 and 0.53, respectively), as well as in F1 in aged extracts, were observed for the HPLC-pHloroglucinolysis and MCP tannins [Table S5(C)].

Regarding HPLC-pHloroglucinolysis parameters, Figure 3 illustrates similar trends for the mDP and % galloylation within fractions (F1-F4) across most vintages. Firstly, mDP ranges were reported for each fraction: F1 (2.06-2.64), F2 (2.82-5.07), F3 (2.8-5.36) and F4 (2.57-3.78). In older wines, apart from the 2004 and 2009 vintages, mDP increased from F1 to F2, then decreased towards F3. On the other hand, besides 2012, there were successive increases in mDP as a function of fraction, from F1 to F3, especially within younger wines. Generally, the increases in mDP in young and aged wines are minimal for most of the fractions across the same sample. Moreover, mDP values decreased as a function of wine age within F3, indicating that younger wines within F3 were characterised by higher mDP values than older wines. Fractions F4 showed a similar effect, yet this was not as pronounced.

Mass conversion percentages were calculated for all extracts and compared to mDP. Including all vintages, MC% values increased in the order F1 (2.23-13.39 %), F3 (5.24-17.43 %) and F2 (4.27-18.91 %). Generally, MC% decreased with wine vintage, and higher MC% were obtained for younger wines (2011-2018). Lastly, for the degree of galloylation (expressed as %), the following ranges were obtained for F1-F4: F1 (2.56-6.05), F2 (3.52-6.24), F3 (4.7-7.25) and F4 (4.09-6.87).

As observed in Figure 3, % galloylation increased from F1–F3 across most vintages. Finally, the average molecular mass showed similar trends to that of mDP, as values for average molecular mass increased with a fraction (F1–F3) across most vintages. Additionally, it appeared as if molecular mass decreased as a function of wine age, especially for F3. For effects according to wine fraction, within most older vintages (2003-2010), average molecular mass was highest for F2, whereas within most younger wines (2011-2018), average molecular mass steadily increased along fractions F1-F3 instead. Across all fractions, younger vintages possessed higher average molecular masses than older vintages.

9. Sensory analyses

Figure 4 illustrates a plot of mean sensory scores obtained from 16 panellists for astringency and bitterness in relation to vintage year. A strong positive correlation (r = 0.8) was observed between astringency and bitterness scores. Although differences were observed for the 15 evaluated wines (Figure S1), no trends were observed across vintages. The significance of the statistical test performed might be
due to some of the wines showing high or low scores for both attributes.

10. Multivariate statistics

10.1. Multivariate analyses of chemical data using PCA

Having already discussed initial findings based on trends within tabulated data, it was beneficial to identify potential intercorrelations between the chemical variables first and how they may change as a function of wine age.

The first principal component accounts for 36% of the explained variance (Figure 5). A clear distinction can already be made with the separation between newer and older vintages within PC1. Apart from the 2009 and 2010 vintages, it appears as if the older wines are more closely associated with higher proportions of galloylation in F2 and F4. Contrastingly, apart from the 2011 and 2012 vintages, the younger wines were generally characterised by higher mDP values, the proportion of epicatechin, as well as average molecular weight across all


FIGURE 5. PCA plot for the first two PCs, containing the most relevant and significant chemical variables within the present study.
their fractions, in addition to catechin (within F2 and F4) and MCP-determined tannin concentrations (for fractions of F2). For PC2 (20 % of the explained variance), while no trends were observed across vintages, a positive correlation was observed between tannin estimation methods (namely, MCP and HPLC) across all classes (besides MCP tannin in F1, and to a lesser extent in F2). Moreover, 2012 and 2018 were characterised by high proportions of galloylation within their respective F3 fractions.

Figure 6 shows a PCA plot to visualise the explained variance of the third component. PC3 (10 % of explained variance) accounts for MCP tannin (in F1), as well as the proportion of epicatechin gallate and tannin activity values (both within the F2-fraction). Ultimately, the three PCs accounted for 66 % of the explained variance.

10.2. Multifactorial analyses of chemosensory data
As one of the aims of this research chapter is to evaluate how the integration of chemosensory variables changes according
to wine age, wines grouped together may highlight similarities within their chemosensory profiles. This was accomplished by additional multifactorial analyses using individual factor maps and correlation plots (Figures 7 and 8).

Comparing how chemosensory attributes change according to wine age, vintages 2016-2018 were strongly and positively associated with astringency. Low positive associations were observed in the 2006, 2010 and 2013 vintages, while negative correlations with astringency existed within the 2004, 2005, 2008 and 2011 vintages (Figure 7 and 8). Furthermore, wine vintages in 2012 and 2006 were positively associated with bitterness, while vintages in 2009, 2011, 2014 and 2015 were inversely correlated with bitterness.

From Figure 8, 11 chemosensory interactions comprising primarily of F3 and F4-fractions were chosen based on their Pearson correlation coefficients to establish relationships between astringency and bitterness. All chemosensory variables possessed r-values higher than 0.5, except for two chemosensory interactions, being that of tannin activity in F3 for either astringency or bitterness, both of which had low negative associations (r = -0.27 and -0.30, respectively). Contrastingly, a moderately positive correlation was observed between the percentage of galloylation within F3 and bitterness (r = 0.54). Apart from the aforementioned variables, all selected chemosensory variables were negatively correlated with one another, and include that of tannin estimation methods using either RPLC (for F3, \( r_{\text{astringency}} \) and \( r_{\text{bitterness}} \) = -0.59 and -0.51, respectively; F4, \( r_{\text{astringency}} \) and \( r_{\text{bitterness}} \) = -0.63 and -0.51, respectively) or MCP (for F4, \( r_{\text{bitterness}} \) = -0.45), proportions of epicatechin gallate (in F4, \( r_{\text{astringency}} \) and \( r_{\text{bitterness}} \) = -0.75 and -0.63, respectively) and catechin (F4, \( r_{\text{bitterness}} \) = -0.53).

**DISCUSSION**

Some authors have used large sample sets with wines of different regions, cultivars and vintages in attempts to understand potential structure-activity relationships (Barak and Kennedy, 2013; Revelette et al., 2014; Watrelot et al., 2016; Yacco et al., 2016). In all cases, low positive correlations were obtained between tannin activity and concentration, which suggested that other factors also played an important role in modifying enthalpy. Indeed, Yacco et al. (2016) found that molecular mass is a key factor in modifying tannin activity values, in addition to tannin oxidation and polymeric pigmentation. To this end, single-vineyard Pinotage wines ranging from multiple vintages were chosen to minimise variation caused by cultivar or region. It was thus thought that wine age as a function of vintage would give an indication of how tannin activity is influenced over the extent of the ageing process. In addition to this, purified molecular mass fractions would provide additional information to understand the tannin structure-activity relationships better.
For the first time, positive activity values were obtained for a few vintages, primarily in the F1-fraction. It is thought that these values may be related to additional low molecular weight compounds such as polymeric pigments and tannin derived monomers and dimers, which may contribute to the endothermic, positive enthalpy values. However, additional techniques such as thin-layer chromatography or mass spectrometry (Kennedy and Taylor, 2003) to qualitatively profile the fractions and obtain tannin structural information were not applied in this study and the above reasoning could not be analytically proved.

In most cases, activity values increased across fractions from F1 to F3, as observed previously (Barak and Kennedy, 2013). With F4 constituting equal aliquots of each of the previous polymeric fractions, it was therefore expected that activity values for F4 would have an intermediate, average value per sample. However, this was not the case for most of the wines. In this regard, the moving average trendline of F4 illustrates that activity values do not follow a clear trend as wine ages. This variation may be attributed to seasonal changes during the growing season, which may affect grape maturity (Ferrero-del-Teso et al., 2020a), as well as winemaking operations (Ma et al., 2018; Revelette et al., 2014; Yacco et al., 2016), all known to modify tannin activity values. Despite this, all fractions (F1-F4) within older vintages (2003-2008) generally had lower tannin activity values than younger wines (2011-2018), in agreement with Barak and Kennedy (2013) and Yacco et al. (2016). Lower redox potentials of high molecular weight oligomers (which oxidise easier than tannins of lower molecular weight) (Li et al., 2008), low mass conversion rates of older proanthocyanidin samples as determined by phloroglucinolysis (Yacco et al., 2016), potential acid-catalysed cleavage reactions, decreased tannin polymerisation reactions due to steric effects (Cheynier et al., 2006) as well as tannin precipitation (Teng et al., 2019, 2021) might explain the results observed.

Subunit composition and abundance across vintages were also considered, given their relevance in tannin chemistry and astringency perception. In the present study, subunit levels of catechin, epicatechin and epicatechin gallate generally decreased as a function of wine age. Multiple studies have investigated the effects of age on phenolic composition, finding similar results where subunits catechin and epicatechin decrease as a function of ageing, in most cases, indicative of polymerisation and condensation reactions (Balga et al., 2014; Cassino et al., 2019; McRae et al., 2012; Stavridou et al., 2016). Additionally, range differences between the minimal and maximal values for catechin and epicatechin decreased in the order from F1 to F3, which allude to increased structural modifications across each polymeric proanthocyanidin fraction.

The mDP results can be summarised as follows, similarly to Quijada-Morin et al. (2012): F1 consisted mostly of dimers, and trimers in younger vintages, while F2 and F3 contained tannins between the tri- and pentameric levels. In fact, pentamers were most prevalent within the younger vintages of F3. The decrease in older vintages on mDP values within F3 may demonstrate the potential effect of tannin oxidation. This is similar to research performed by Barak and Kennedy (2013), who reported on lower mDP values in aged red wine extracts (min. mDP = 2.6) relative to that of newer wines (max mDP = 38.3). The low mDP range (between 2 and 5) of extracts in the present study can also be attributed to their lower mass conversion rates, which decrease as a function of wine age. This can be explained by the incomplete depolymerisation of proanthocyanidic material into known subunits, as intramolecular bonds, resistant to cleaving by phloroglucinolysis, are formed. Secondly, findings by Esatbeyoglu et al. (2011) suggest that also interflavonoid bond location may also affect conversion yield, as C₆-C₈ bonds are more easily cleaved than that of a proportion of C₆-C₆ within an intact proanthocyanidin, which would remain uncleaved. Lastly, the molar absorptivities of flavanols (such as epicatechin-phloroglucinol, epicatechin-gallate and its phloroglucinol adducts) and polymeric pigments (Kennedy et al., 2006) differ considerably (Kennedy and Jones, 2001) which lead to inaccurate estimates of subunits. In the present study, HPLC-phloroglucinolysis-determined tannin concentrations and conversion rates are theoretical, as they are only based on catechin and epicatechin standards and their adducts. Consequently, these concentrations are underestimated and do not reflect their true tannin concentrations. More accurate quantification would require epicatechin gallate and epigallocatechin standards (both of which were unavailable), along with their phloroglucinol adducts. In the first case, the abundance (in nmol) of epicatechin gallate and its adduct was based on a previous calibration series (where all subunits and adducts were generated), which automatically integrated both of their defined and well-resolved peaks. The previous calibration series made it possible to obtain the abundance in nmol of epicatechin gallate, even in the absence of the required calibration standard. This explains why they are used in the calculations related to subunit composition but not tannin concentration. In terms of epigallocatechin, the absence of a calibration standard made quantification of itself and its phloroglucinol adduct difficult, which was compounded by the fact that epigallocatechin was poorly resolved from other peaks, even from previous calibration chromatographs. Based on this, epigallocatechin and its phloroglucinol adduct were not used in any further calculations related to both subunit composition, mass conversion or tannin concentration, which might result in underestimation of these parameters, given that epigallocatechin levels have been reported to account for 20-30 % of the tannin concentrations, as noted in several cultivars in other previous studies (Quijada-Morin et al., 2012; Arapitsas et al., 2021). Regardless, it was assumed that because of the use of a single cultivar and the similar winemaking practices applied across vintages, the error committed among samples may be negligible.

Importantly, the present study contains low molecular-weight fractions (found mostly in F1) containing monomer-derived tannins, where phloroglucinolysis may result in decreased
mDP without any losses in tannin concentration (Vidal et al., 2002). This reflects the importance of tannin origin, age and ageing reactions in the assessment of phloroglucinol-based parameters (McRae et al., 2012). Relating mDP to oxidation, it was shown that high molecular weight proanthocyanidins have lower redox potentials than oligomers and are, therefore, more easily oxidised (Li et al., 2008). The lowest and highest range values for mDP were obtained for F1 and F3, respectively. This suggests that the highly polymeric F3 fraction contains the highest amount of variation. An important consideration is that oxidation over long-term ageing periods is also likely, given that these wines were stored using cork closures.

Similarly to mDP, molecular mass also decreased in older wines, especially within F3, which suggests that age-related reactions such as cleavage reactions and oxidation play an important role in modifying wine chemistry over time (Bindon et al., 2014). Lastly, the increase in % galloylation from fraction F1-F3 (Barak and Kennedy, 2013; Prieur et al., 1994) can be attributed to the incorporation of galloyl-derivatives from seed tannins during wine ageing, which then polymerises further with time. However, the correlation may not be indicative of a cause. Other factors may also affect the chemical evolution of phenolic compounds, such as grape origin and oenological parameters, such as maceration and maturation duration, and barrel ageing regimes (Barak and Kennedy, 2013; Stavridou et al., 2016; Yacco et al., 2016), which are indicative of seasonal differences between vintages.

Multiple reviews based on red wine phenolic compounds are available where correlations between methods of tannin estimation are evaluated (Alexandré-Tudo et al., 2017; Cáceres-Mella et al., 2013; Wilhelmy et al., 2021). In the present study, tannin concentrations were determined using either HPLC-PLRP, HPLC-phloroglucinolysis or MCP. In agreement with Watrelot (2021), a strong correlation was found between the total HPLC-PLRPs and MCP tannin concentration, as observed across fractions, especially in younger wines. It appears as the correlation was found to be dependent on both wine age and wine fraction (indicative of molecular mass). Despite both methods being measured at 280 nm, the HPLC method for measuring tannin content is based on peak areas, while MCP is based on precipitation of a tannin-protein complex. The low correlation obtained within the older vintages may indicate that MCP may react similarly to some colorimetric assays such as the vanillin (Sun et al., 1998) or DMAC assay (Wallace, 2010) in that its reactivity and ability to precipitate tannins decrease as a function of mDP. Yet, more research is required to prove the validity of this statement.

In addition, poor correlations were observed between HPLC methods. While PLRP accounts for the entire tannin peak area eluting between the area after epicatechin and just after partial tannin as found in its respective chromatograms, phloroglucinolysis integrates and quantifies subunits and their adducts using calibration standards. This later method does not account for subunits not quantified due to phloroglucinol-resistant bond formation. Moreover, in this study, only catechin, epicatechin and their adducts were quantified with certainty and additional standards may result in higher positive correlations, but this is speculative and requires further investigations.

Lastly, phloroglucinolysis-determined tannin levels were lower than that of MCP (almost a 10-fold difference in magnitude). This contrasts with Seddon and Downey (2008), where tannin levels by phloroglucinolysis were higher. This could be explained by sample type, where the current study utilises aged wine extracts, the reported study used skin extracts, which would theoretically have higher mass conversion rates and lower condensed tannin levels due to limited structural modifications. Poor correlations were observed in most sets except F3 for younger wines extracts. The MCP assay is selective for condensed tannins, which would explain why higher correlations would exist in the F3 fraction. This could also be due to younger wine extracts being less structurally modified, leading to fewer structural modifications, which would otherwise underestimate tannin content as determined by phloroglucinolysis.

Finally, a decrease across periods of long-term ageing was not observed in this study. In this regard, it is critical to consider the vintage effect across seasons, where multiple viticultural (i.e., water stress or grape ripeness) and oenological factors (i.e., maceration, oxidation or fining) may result in wines of distinctive phenolic profiles. This may explain why aged wine extracts possess higher tannin concentrations than younger ones whilst remaining cognizant of the effects of factors such as oxidation, precipitation and polymerisation reactions across periods of long-term ageing. Additionally, the present study illustrates that each assay has its unique way of interacting with a sample, which appears highly dependent on age, as this introduces structural modifications which affect the assay response and the accuracy thereof.

Sensory analysis was also performed to establish potential relationships with measured chemical variables. Positive correlations were observed between the two mouthfeel attributes, as previously reported (Miheea et al., 2018; Sáenz-Navajas et al., 2019). Additionally, their mean scores show that astringency scored higher than bitterness across all vintages, which suggests that participants were able to distinguish between these attributes. With respect to wine age, no trends were observed, indicating that viticultural/oenological practices associated with seasonal changes play an important role.

PCA was also used to identify additional relationships between chemical variables. Among important findings within wines of newer and older vintages, the latter were characterised by higher proportions of galloylation than newer vintages in all fractions, except for the F1-fraction. Newer vintages also contained higher proportions of catechin and epicatechin subunits than older vintages, which suggests that younger wines are undergoing structural modifications, which are more advanced in older wines, where these
subunits are prone to age-related reactions such as oxidation (Poncet-Legrand et al., 2010; Yacco et al., 2016) and even precipitation (Teng et al., 2019; Teng et al., 2021).

Most authors use chemosensory studies, with chemical analysis performed on wines or entire extracts in model wine solutions, followed by sensory analyses for specific attributes. However, within this study, fractionated extracts of a given red wine were used for chemical analysis instead, and each fraction was compared to individual sensory attribute scores obtained from the sensory panellists of the original wine. Therefore, the red wine extracts do not contain the same matrix as intact red wines, which also differs considerably from fractionated extracts. This may also explain the negative correlation and unusual response obtained between the red wine mouthfeel attributes discussed and chemical parameters such as tannin concentration.

With tannin activity, a low negative correlation existed between tannin activity and both bitterness and astringency. This indicates a linear positive association, given that ΔH is a negative, exothermic reaction (Barak and Kennedy, 2013; Watrelot et al., 2016). This is because the activity data used for PCA and MFA was performed using their negative values. Therefore, the F3-fraction was positively associated with astringency and bitterness, which suggests that highly polymeric proanthocyanidins with greater activity values may play a greater role in astringency and bitterness. Both known and unknown interactions may modify the relationship between activity and astringency and include matrix effects such as pH, ethanol and sugar (Fontoin et al., 2008; Watrelot et al., 2016), age-related factors such as polymerisation, oxidation and precipitation (Teng et al., 2019; Yacco et al., 2016), and even the nature of the analyte (crude or purified). Positive associations between higher activity values and increased astringency perception are reflected by the findings of Sáenz-Navajas et al. (2019). Ultimately, it appears as though the higher oligo- and polymers play a greater role in affecting the chemosensory relationship between activity and astringency.

CONCLUSION

The effect of wine age and tannin composition was investigated in a single red wine across multiple vintages using a chemosensory approach. The results obtained showed that tannin activity values were driven primarily by molecular mass, especially within highly polymerised samples, as opposed to wine vintage. Low molecular weight tannins (F1) were associated with positive activity values for the first time, suggesting a different interaction mechanism than the highly exothermic reaction between polymeric proanthocyanidins (F2 and F3) and salivary proteins. While poor correlations existed between tannin activity and concentration (as determined by HPLC-PLRPs), generally low and moderately positive correlations were observed between all the tannin estimation methods. A decrease in activity values across all wine fractions (F1-F3) in older vintages may be attributed to age-related reactions, including cleavage reactions, oxidation and tannin precipitation, which illustrates that concentration is one of many factors influencing tannin activity over time. Tannin activity is affected by multiple viticultural and oenological factors and modified from the onset of berry development, throughout fermentation and maceration, to periods of long-term wine ageing and storage. Additional chemosensory analysis was performed using a multivariate approach to understand how activity values are related to bitterness and astringency. While tannin activity values across all fractions explained little variation, it was found that large, polymeric proanthocyanidins, associated with more negative (exothermic) activity values, were positively associated with astringency and bitterness. The results of this study suggest that more research is required to evaluate the effect of proanthocyanidin size on the chemosensory relationship between tannin activity and mouthfeel properties.

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