Colour in Pinot Noir wines:

does climate have an impact?

A thesis submitted for the degree of Master of Philosophy

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Abstract

Climate change is an increasingly important issue that will affect agriculture, with increasing temperatures being one of the most noticeable impacts. Viticulture is particularly sensitive to climate change, since climate forms a key part of the *terroir* of a vineyard or a region. In particular, *Vitis vinifera* L. 'Pinot Noir' is known to be sensitive to the temperature in which it is grown, and does not grow well in hot climates.

Consequently, a trial was run over two years in a commercial vineyard near Lenswood, in the Adelaide Hills region of South Australia, to determine the impact of higher temperatures on the Pinot Noir grape and wine colour. The study used a passive over-the-vine heating system, which had previously been trialled in the Barossa Valley, South Australia.

As expected, the study found that elevated temperatures had decoupled the accumulation of sugars and anthocyanins in grape berries, with the heated treatment grapes producing lower anthocyanin levels than the control treatment grapes per gram of total soluble solids. The temperature effect was less defined for the wines than for the grapes, with the wines from the heated treatment vines having higher total phenolics, and perhaps as a consequence lower CIELab b* values (i.e. less blue pigmentation).

Two small scale fermentation trials were also undertaken. One investigated the use of Bodum coffee presses for small scale, replicated fermentations. It also examined whether the position on a slope would influence wine colour and hence be a confounding factor for studies that examine wine colour. It found that the coffee

presses functioned satisfactorily as fermenters, and that location on a slope is not necessarily a confounding factor. This latter finding in particular strengthens the results of the vineyard heating trial detailed above, where this was a potential confounding factor.

The second fermentation study examined the effects on wine colour of inclusion of stems in ferments, a popular if controversial technique for Pinot Noir. As expected, wines made using stems were higher in tannins and darker in colour, appearing more brick red than wines made without stems.

Finally, inductively coupled plasma mass spectrometry (ICP-MS) and nuclear magnetic resonance (NMR) spectroscopy metabolomic techniques were used to characterise a variety of Pinot Noir wines from selected southern Australian wine regions, with wine from Central Otago, New Zealand, used as an out-group. ICP-MS showed promise in determining regionality in Australian and New Zealand wines, with rare earth metals proving particularly useful. NMR analysis of metabolites was less useful, but may be informative for studies of winemaking and viticultural techniques. NMR was expected to be able to distinguish between vintages, but this was not achieved.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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In memory: Sophie Christofis; John Earl.

Chapter 1. Literature Review

1.1 Introduction

Vitis vinifera L. 'Pinot Noir' is the fourth most commonly planted red grape cultivar in Australia (Halliday 2011) with 4,980 ha producing 34,600 tonnes in 2012, 13,700 tonnes of which were produced in warm inland regions (e.g., the Riverland, which accounted for 10 % of the total production of Pinot Noir), and 20,800 in cool climate regions (e.g., the Adelaide Hills, which accounted for 15 % of total production)[1] (Wine Australia 2012). By 2016, 4,950 ha were producing 43,200 tonnes, with 17,500 tonnes from warm inland regions (with the Riverland accounting for 16 % of production, and the Murray Darling and Riverina for 10 % each), and 25,800 tonnes from cool climate regions (with the Adelaide Hills and the Yarra Valley both accounting for 11 % each of production) (Wine Australia 2016).

It is likely the majority of Pinot Noir produced in Australia is used in sparkling wines (Halliday et al. 2015). However, due to its perceived reputation as one of the most heavily sought-after of all the grape cultivars amongst wine aficionados, its status in fine wine outweighs its position in terms of area planted. One of the chief difficulties in producing a high quality wine from Pinot Noir is colour extraction. Pinot Noir grapes are thin skinned, and low in tannins and anthocyanins (Dambergs et al. 2012; Nanson 2005-2012; Rowley 2015), the compounds which are responsible for the colouration of skins in red and rosé (*gris*) cultivars (Revilla, E, Ryan & Martín-Ortega 1998).

To quote Pool (2000, para. 5):

[1] Where the additional 100 tonnes not accounted for in this breakdown was sourced from is not documented.

> "*Insufficient color is often a problem with Pinot Noir, but even where color is lacking it is valued for the production of white sparkling wines as in Champagne…. Regardless of where it is grown, only a small fraction of Pinot Noir wines achieve the ultimate in color, intensity of flavor and body which characterize the grand crus, but everywhere that sound grapes can be grown, Pinot Noir produces wines which are regional favorites.*"

Consequently, consumers are typically resistant to lightly coloured red wines, perceiving them as lower in quality than darker red wines (Parpinello et al. 2009). For example, wine critic Richard Hemming (Hemming & Rosen 2015) noted that the consumer's first impressions of the wine will be based on its colour, and that

> "*Opaque reds naturally evoke an expectation of concentrated flavour, and perhaps higher tannin and alcohol*" (Hemming & Rosen 2015, paragraph 5),

although he did note that wines may not comply with these preconceptions.

In contrast, Valentin et al. (2016) found minimal support for any positive relationship between perceived wine colour and perceived quality for Pinot Noir wines, at least amongst French and New Zealand wine professionals. This was backed up by wine critic Jamie Goode, who noted that there has been an increase in interest in light red wines, including lighter coloured Pinot Noirs, among wine consumers over the past few years (Goode 2015). Goode notes in particular that:

> "*wine lovers are beginning to realize that there is no correlation between depth of colour and wine quality*"(Goode 2015, paragraph 6)…
>
> "*Pinot Noir was never meant to make dark red wines… Pinot is about elegance and aroma*" (Goode 2015, paragraph 7).

Hemming (Hemming & Rosen 2015) also noted a shift in the market in Australia, France, and the USA towards lighter, less extracted wines, and hence less importance being placed on colour density. However, this trend has not been noted in China, an important emerging market for Australian wines.

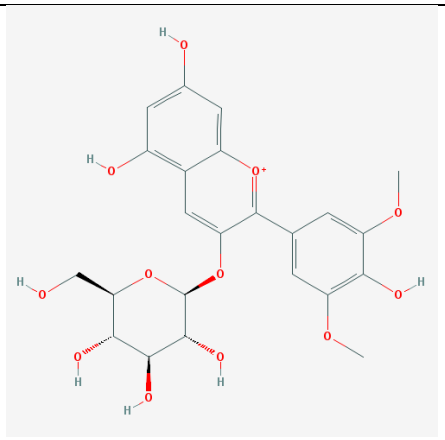
However, Dambergs goes as far as to state that

"*there is no doubt that tannin and pigment determine quality in Pinot Noir*" (Dambergs et al. 2012, p. 18)

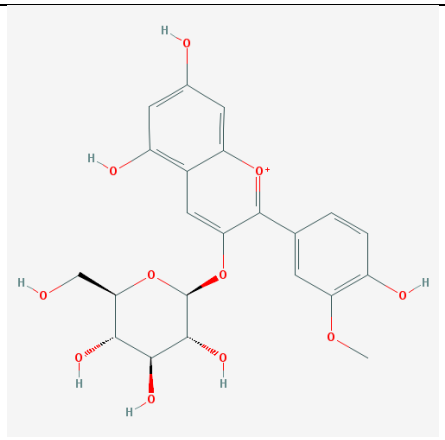
In support of this opinion, he noted that an Australian Wine Research Institute (AWRI) analysis of wines entered for the two year old Pinot Noir category of the Tasmanian wine show demonstrated that gold and silver award winning wines had high pigment and tannin concentrations (Dambergs et al. 2012). Hence, colour extraction is of interest to fine wine producers.

1.2 Pinot Noir anthocyanins: structure and analysis

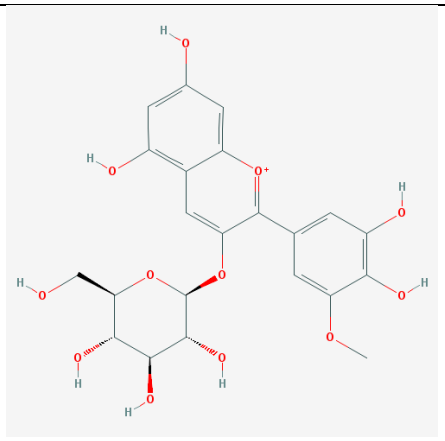
Anthocyanins are the pigments responsible for the colouration of red and pink ('*gris*') grapes. They belong to the flavonoid family, and are glycosylated derivatives; anthocyanidins are the corresponding aglycones. Anthocyanin accumulation begins at *véraison*; however, concentrations may drop towards the end of the ripening period, particularly in hot climates. It is possible that anthocyanins may be converted to other pigments (Cheynier et al. 2010).



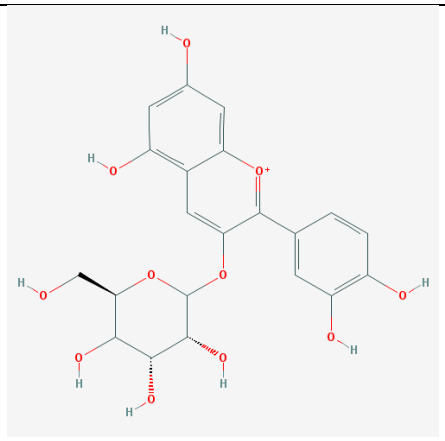
a. Malvidin 3-monoglucoside



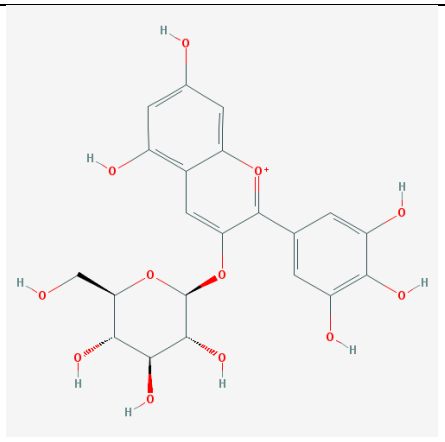
b. Peonidin 3-monoglucoside



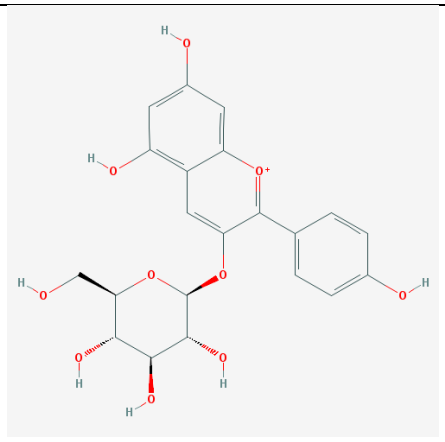
c. Petunidin 3-monoglucoside



d. Cyanidin 3-monoglucoside



e. Delphidin 3-monoglucoside



f. Pelargonidin 3-monoglucoside

Figure 1.1: Structures of anthocyanins in monoglucoside forms. Retrieved from PubChem Open Chemistry Database (Kim et al. 2016)

Only five anthocyanins (see Figure 1.1) had generally been considered to occur in Pinot Noir: Malvidin 3-monoglucoside (a), Penonidin 3-monoglucoside (b), Petunidin 3-monoglucoside (c), Cyanidin 3-monoglucoside (d) and Delphinidin 3-monoglucoside (e). In 2010, the presence of pelargonidin-3-O-glucoside (f) was confirmed in Pinot Noir (He et al. 2010; Lorrain et al. 2013). However, Pinot Noir does not contain any acylated anthocyanins.

As with other wine grapes, its anthocyanins are simpler, and therefore more reactive, than those found in flowers (Figure 1.2) (Brouillard, Chassaing & Fougerousse 2003).

Identification and quantification of anthocyanins can be undertaken using high performance liquid chromatography (HPLC or LC) (Santos-Buelga, Mateus & De Freitas 2014; Welch, Wu & Simon 2008) in combination with diode array detection (DAD) (Boutegrabet et al. 2011; Peng et al. 2001; Peng et al. 2002; Roullier-Gall, Lucio, et al. 2014; Versari et al. 2014), with mass spectrometry (Cheynier et al. 2010; de Villiers et al. 2011; Santos-Buelga, Mateus & De Freitas 2014), or with nuclear magnetic resonance (NMR), e.g. solid phase extraction and LC with NMR (Santos-Buelga, Mateus & De Freitas 2014). These techniques can be applied both to grape skin extract and wine.

One such method is outlined in Peng et al. (2002) & Cozzolino et al. (2004). This method used HPLC-DAD with a reverse phase C18 column. Boutegrabet et al. (2011) proposed a method using UPLC (ultra-high pressure liquid chromatography) to determine phenolic compounds (including, but not limited to, anthocyanins) in wine.

A number of detectors have been used in combination with HPLC, including single or multiple wavelength UV-Vis, DAD, which can scan a range of wavelengths including UV to produce spectra, fluorescence detection, and mass spectrometry (MS). UV detection remains the most important, as phenolic compounds are strongly absorbent in the UV region (Lorrain et al. 2013).

While most HPLC methods use reversed phase methods, one study suggested using normal-phase chromatography for the analysis of higher molecular weight proanthocyanidins (Kennedy & Waterhouse 2000; Lorrain et al. 2013). Similar work has also been done using a novel mixed-mode ion exchange reversed phase column (Lorrain et al. 2013; Vergara et al. 2010).

Complex analytical techniques have come to include tandem MS (MS/MS) usually in conjunction with LC or with gas chromatography (GC), and matrix-assisted laser desorption/ionisation combined with time-of-flight MS (MALDI-TOF). These have been used, sometimes in conjunction with NMR, to determine the structure of phenolic compounds (Flamini 2003; Lorrain et al. 2013; Santos-Buelga, Mateus & De Freitas 2014; Wang, Kalt & Sporns 2000).

Simple visible spectroscopy has also been widely used to quantify the concentration of anthocyanins in grapes and wine. The Iland method (Iland et al. 2004, pp. 44-48; Iland et al. 1996) is used for grapes, and Somers Analysis (Mercurio et al. 2007; Somers 1971; Somers & Evans 1974, 1977) is used for wine. In both cases, a measure of total anthocyanins is provided, and individual anthocyanins are not identified or quantified. Further details on these methods can be found in Chapter 2.

1.3 Viticulture

> "*Grape anthocyanin composition also depends on the maturity and vine growing conditions including soil and climate characteristics but also practices such as pruning, fertilization, or watering*." (Cheynier et al. 2010, p. 1138)

1.3.1 Region and 'terroir'

Since Pinot Noir is grown in a range of different climates across Australia, it would be beneficial to discover whether viticultural climatic conditions can impact colour in the finished wine. It would also benefit the Australian wine industry to be able to identify the most suitable sites to plant this cultivar, and to quantify the potential of existing plantings for the production of high quality wines. It will also be particularly critical to know the impact of higher temperatures on the production of Pinot Noir wines, especially due to climate change. While such changes will be detrimental to warm climate regions, it may allow grape production in regions previously considered too cold (Jones & Schultz 2016; Schultz & Jones 2010).

The environment in which the grapes are grown has an impact on the characteristics of the grapes and wine. Factors such as climate, soil, and geology are all considered important. This interaction between vine and environment is referred to by the French as '*terroir*' (Barnard 2016; Hong 2011; Kelebek et al. 2010; Mignani et al. 2014; Riovanto et al. 2011; Roullier-Gall, Boutegrabet, et al. 2014; Roullier-Gall, Lucio, et al. 2014; Son et al. 2008; van Leeuwen et al. 2004; van Leeuwen & Seguin 2006). Seguin defines terroir as:

> "*an interactive ecosystem, in a given place, including climate, soil, and the vine (rootstock and cultivar)*" (Seguin 1988, cited in van Leeuwen et al. 2004, p. 207).

Roullier-Gall, Boutegrabet, et al. (2014) state that

> "*Historically, terroir refers to an usually rather small area whose soil and microclimate impart distinctive qualities to food products […] and it is now accepted that metabolites in wine could be affected by the terroir [….] Terroir is the greatest determining factor of all great wines*" (Roullier-Gall, Boutegrabet, et al. 2014, pp. 100-101).

Barnard (2016) remarks on the links between the words 'terroir' and 'territory', and that

> "*This territory possesses certain homogeneous physical properties (soil, geology, drainage, etc.) which are suitable for certain agricultural products. Specifically, the nature of the soil is thought to be communicated through the character of certain products, most notably with wine. Wine, according to its terroir, will have a particular taste that is associated with the soil, geology, topography, and climate where the vineyard is located.*" (Barnard 2016, pp. 14-15)

More simply, terroir can be thought of as, "the taste of the place" (Barnard 2016, p. 15). However, as Bessis, Fournioux and Jeandet (1996, p. 129) note,

> "*Discussions on the alchemy of typicity are very far from over*".

There have been numerous studies that demonstrate sensory differences between wines from different geographical origins (Imre et al. 2012; Nicholas et al. 2011; Robinson, A et al. 2012; Seguin 1986; Tomasino 2011). One study (Robinson, A et al.

2012) looked at Cabernet Sauvignon wines from different geographical indications within Australia, and discernible differences were noted. Another such study (Guinard & Cliff 1987) was able to demonstrate that Pinot Noir wines from Carneros were unique within California. A further study (Tomasino 2011) undertook to characterise regional examples of New Zealand Pinot Noir, and was successfully able to differentiate between the main regions based on a study involving both a tasting panel and chemical analyses, using a method developed specifically for New Zealand Pinot Noir, namely Headspace Solid-phase Microextraction combined with GC-MS.

A number of studies on the *terroir* effect have been undertaken by Régis Gougeon's lab at the Université de Bourgogne in Dijon, France. In one (Roullier-Gall, Lucio, et al. 2014), targeted UPLC and Fourier-transform ion cyclotron resonance MS (FTICR-MS) were used to attempt to chemically differentiate between grapes, must, and wine from two Pinot Noir vineyards in Burgundy that are owned by the same *domaine* (wine estate). The phenolic profiles for were found to be quite distinct for each area, within a given vintage. These differences are notable in grapes, must, and wine. A similar study (Roullier-Gall, Boutegrabet, et al. 2014), this time looking at four different vineyards, found similar results.

1.3.2 Climate

The importance of climate is noted in a number of sources (Aghemo, Albertino & Gobetto 2011; Butler 1981; Gladstones 2011; Roullier-Gall, Boutegrabet, et al. 2014; Roullier-Gall, Lucio, et al. 2014; Rowley 2015; Sadras & Morán 2012; Sadras, Petrie & Morán 2013; van Leeuwen et al. 2004; van Leeuwen & Seguin 2006; Webb, Whetton & Barlow 2008). Pinot Noir generally is thought to make uninteresting wines in hot conditions, with the resulting wines lacking in colour (Antcliff 1976;

Butler 1981; Galet 1979; Robinson, J & Harding 2015), with the *Oxford Companion to Wine* noting that Pinot Noir performs best

> *"on calcareous soils and in relatively cool climates where this early-ripening vine will not rush towards maturity, losing aroma and acidity"* (Robinson, J & Harding 2015, par. 5).

However, in North America good Pinot Noir wines have been produced in warmer Californian regions such as Rutherford and Dry Creek. It may be that particular sites in these regions are suited to Pinot Noir due to factors such as altitude and aspect that compensate for the warmer climate (Haeger 2004). In Australia, Pinot Noir has been successfully grown in the Hunter Valley, NSW, often on *argilo-calcaire* (clay-limestone) soils (Graham 2012; Halliday 2006). However, it is worth noting that these conclusions are not the result of specific scientific studies, but instead are presumably the result of the authors' anecdotal experiences.

Colour in grapes has been linked to viticultural climate, using studies conducted on potted vines in greenhouses. The impact of temperature on grape colouration in Pinot Noir in particular has previously been examined using potted vines grown in greenhouses (Kliewer 1970; Kliewer & Torres 1972). However, the studies cited were limited in that they were conducted on a limited number of vines grown in pots in phototron rooms with analysis conducted on berries only, not on a finished wine. However, both studies noted a decrease in colour (in terms of optical density measured at 530 nm) in grapes grown in warmer conditions. Another study looked at colour in a hybrid *Vitis* cultivar, *Vitis labrusca* × *V. vinifera* 'Aki Queen' (again, using potted vines grown in greenhouse conditions), and noted that anthocyanin accumulation was higher at 20 °C than at 30 °C (Yamane et al. 2006).

This effect was later demonstrated again with a non-hybrid 'classic' variety, *Vitis vinifera* 'Cabernet Sauvignon' grown in pots in a phytotron (Mori et al. 2007). In this study, they noted that at higher temperatures (defined as daytime maximum 35 °C, nighttime maximum 20 °C), anthocyanin levels in grape skins were less than half that of those grown in the control conditions (defined as daytime maximum 25 °C, nighttime maximum 20 °C). Anthocyanin levels were determined via HPLC. This study attributed a number of potential causes, including chemical and/or enzymatic degradation of anthocyanins.

There may be a correlation with the Burgundy region, as the south of Burgundy is warmer than the Côtes de Nuits, where the most distinctive wines of the region are produced (Butler 1981). However, some of the colder sites of the Côtes de Nuits also appear to produce wines that are lower in colour than more favoured sites, e.g., Marsannay, one of the colder appellations in the Côtes de Nuits, *versus* Vosne-Romanée (GP Duley, personal observations).

From the data available on the Météo-France website (Météo-France 2013), it is possible to compare average temperature, rainfall and sunlight hours for Mâcon and Dijon. Mâcon is in the Mâcon-Villages appellation, and Dijon is just to the north of the Marsannay appellation (Chenôve, a village in the Marsannay appellation, effectively being a suburb of Dijon). It would appear the differences are very subtle, with Dijon being on average around a degree or less cool than Mâcon most months of the year. Rainfall is more variable, with Mâcon receiving ~15 mm more average rainfall than Mâcon in September and October. Interestingly, Dijon also receives slightly less average hours of sunlight than Mâcon during June and July (Météo-France 2013).

It is worth noting, however, that this data does not include adjustments for soil, altitude, aspect, surrounding topography or other aspects of vineyard site which are of major significance (Wittendal 2004). These factors are referred to by Gladstones (2011) as site-temperature adjustment factors. Gladstones uses these factors to provide 'biologically effective degree days', a measurement which can be used to predict harvest date for different grape maturity groups. On the Côte d'Or, these factors may be all that separate a *Grand Cru* site from a *Bourgogne AOC* site.

Based on a study of a range of vineyards in Australia and Burgundy, one study found that highly rated wines from Australia, and various *Grand Cru* and *Premier Cru* vineyards in France, tended to have wine coloration in the range of 75—80 P.T.A. (potential total anthocyanins) (Iland & Fetzmann 2000). Based on an earlier study by Pirie (1977, cited in Iland & Fetzmann 2000), they suggest a temperature range of 17-24 °C for the period between *véraison* and harvest as optimal for the activity of enzymes involved in anthocyanin accumulation (and hence, colour production). Only two sites, both of which fell within their suggested temperature range, were examined in detail, so no comparison with warmer or cooler sites was possible. One of the sites studied was near Lenswood in the Adelaide Hills, South Australia, the other was an unnamed site somewhere in Burgundy (Iland & Fetzmann 2000).

Correlations between climate, quality (as assessed by wine grape colour and levels of glycosyl glucose), and price paid for wine grapes can also be noted. One study (Webb, Whetton & Barlow 2008) examined this correlation in Australia. This paper noted that Pinot Noir is one wine grape where a linear relationship between quality and

climate is displayed, with grapes from cooler climates fetching higher prices, suggesting they are considered to be of higher quality.

On a much smaller scale, one study (Nicholas et al. 2011) looked at the effect of vineyard scale climatic variability (i.e., mesoclimate) on the phenolic composition of Pinot Noir wines. This study examined Pinot Noir grown in a number of commercial vineyards in Sonoma Valley and Los Carneros (California, USA). Some trouble was taken to ensure that the sites were similar, with vines of at least five years of age, and with "similar" clones (Dijon 115 and Pommard 5, except for one site which had UCD 4 and UCD 13).

Several of the findings reported are pertinent to the present study. For example, the heat accumulation in the post-harvest period a year prior to maturity and the heat from bloom to *véraison* correlated negatively with the concentration of anthocyanins, iron-reactive phenolics, and tannins. The authors cautioned, however, that further study was needed to evaluate the causative relationships behind this. They noted a positive correlation between anthocyanin concentration and accumulated hours from 16 °C to 22 °C (similar to the proposed "heat work index", which suggests this temperature range as being ideal for enzymatic activity). In contrast to past research, this paper found a significant increase in skin:berry weight ratio during berry growth. Previous work had shown that the berry grows proportionately, and that phenolic compounds do not change in concentration during ripening (Nicholas et al. 2011).

1.3.3 Soil

The impact of soil on wine colour, and wine quality in general, is still being debated. Fine wines are produced on a wide range of different geological formations and soils

worldwide: there is no single type which is best for wine (Seguin 1986), although in general the best wines come from sites where the soils are well-drained (Haeger 2004).

Nonetheless, some cultivars seem grow best in certain parent materials—e.g., it has been reported that Chardonnay and Nebbiolo are particularly suited to marl, and that Gamay produces excellent wines grown on the schist granite and porphyry of Beaujolais, but does not do as well on Burgundy's marly limestones (Haeger 2004).

Opinion on soil suitability for Pinot noir, in contrast, is mixed. Some authors insist that limestone is essential for good Pinot Noir, noting that Burgundy's soils are predominantly *argilo-calcaire* (clay over limestone) (Haeger 2004; Robinson, J & Harding 2015). Others claim that it does well on "any well-drained loamy mix of sand, clay, and organic matter, as long as it is not too rich or deep" (Haeger 2004, p. 152). It is possible that the defining feature of Burgundy's soils is not their chemistry, but their ability to provide a perfect hydrostatic balance, similar to that found in an irrigated vineyard. Pinot Noir in North America has been noted to have been planted on every kind of soil *except* sedimentary limestone (Haeger 2004).

The impact of soil geochemistry on aroma and chemical profiles of Pinot Noir wines should also be taken into account. One study (Imre et al. 2012) looked at the impact of soil on wines from the Bannockburn region of Otago, New Zealand. The sites selected—at Felton Road Wines, Olssens, and the Mt Difficulty "Target Gully" vineyard—were all within one kilometre of each other, and climatic conditions were assumed to be essentially identical. The vineyards had similar irrigation regimes, were all planted to Pinot Noir 10/5 on own roots, and pruned using the vertical shoot

positioning system. Differences were observed between the sites with regards to wine colour and phenolic composition, with the Olssens wine showing lower colour density than that from Mt Difficulty or Felton Road. This trend was also seen with monomeric pigments and total red pigments, where the Olssens wine again had lower values than the other two sites (Imre et al. 2012).

The same impacts can also be noted with white wines. A study (Gómez-Míguez et al. 2007) which looked at colour and aroma in white wines noted that the type of soil affected the colour of the wines. In this particular study, wines grown on sandy soil showing greater colour intensity than those grown on clay soil. However, these differences, while statistically significant, were not noticeable to a taster. It is also likely that the specific impacts of soil composition will vary depending on the region and the cultivars studied.

These geological impacts can also be observed in Bordeaux, where the impact of soil on wine quality has been noted, in particular as part of the wider concept of *terroir*. Separating out the impact of soil on grape and wine quality from other aspects of *terroir* is difficult. Nonetheless, it has been noted that tasting can reveal differences between wines grown on different soils. One example within Bordeaux is Saint-Émilion, a wine growing village about 40 km east of Bordeaux. It is most famous for its *argilo-calcaire* terroir (van Leeuwen & Seguin 2006), but a number of other soil types are found in the region (Seguin 1986). Here, wines grown on the flat sandy soils have poor colour, and are thin and lacking in finish. In contrast, the wines grown on the Sanoisian calcerous sandstone (which are only a few hundred metres away from the sandy soils) and on the Stampian '*asteies*' limestone both have greater body, elegance, and finesse than the wine from the sandy soils. This is despite the

fact that the same cultivars (mainly Merlot) and the same training system predominate (Seguin 1986).

Seguin (1986) also argued that, given that *Grand Cru* sites are mainly distinguished by their soil, and are known for being able to produce good quality wines from year to year, it must the soil which is the distinguishing feature. Seguin considered that it is the soils of *Grand Cru* sites are able to attenuate harmful climatic conditions such as heavy rainfall or extended periods of drought. This may be due to their physical properties, e.g. structure, macro- and microporosity, as well as root system development—it was noted that the best terroirs have a high degree of macroporosity (e.g., gravel-sand, as found in Bordeaux). Additionally, the soil must also be able to limit yield, as low yields are considered essential for quality wine production (Seguin 1986).

Another study (Wittendal 2004) examined 2,816 vineyards in the Côte de Nuits and Côte de Beaune regions of Burgundy, France. The vineyards included were in Côte de Nuits and Côte de Beaune regions, and were village level, *Premier Cru*, and *Grand Cru* appellations within those two greater areas; vineyards in regional *AOC*s (e.g. *Bourgogne AOC*) were not included. A principal component analysis on geological and climatic data was undertaken. This paper reported that soil type was more important than orientation. Looking in particular at the *Grand Cru* sites that produce red wines, altitude and slope were shown to play only a minor role in defining these particular vineyards from other, less distinguished, sites. More important was the soil, principally colluviums, then limestone. After soil, the orientation was most important, with all *Grand Cru* sites being east facing.

In contrast, van Leeuwen and Seguin (2006, p. 6) noted that while “Some soil types, such as limestone soils, are known for producing generally high quality wines

… throughout the world, outstanding wines are grown on a huge variety of soils." In France's Bordeaux region, for example, great wines are produced on a wide variety of different soils, including heavy clay (e.g., Pétrus and Cheval Blanc), acidic gravelly soil (e.g., Lafite-Rothschild), and alkaline limestone soil (e.g., Ausone) (van Leeuwen & Seguin 2006, p. 6).

Sadras, Bubner and Morán (2012) developed a system to heat vines *in situ* within the vineyard. This system uses panels made of polycarbonate sheeting on steel frames that sit over the vines and heat the vines via a passive heating system. Since different temperature regimes can be trialled in the same vineyard, the impact of confounding factors such as soil, sunlight, or altitude can be minimised (Sadras, Bubner & Morán 2012; Sadras & Morán 2012; Sadras & Soar 2009). This method was used in the present study (see Chapter 2).

1.3.4 Light exposure and phenolics

Light levels (especially vine light interception in the fruiting zone) explain more variability in tannins and total phenolics than vineyard temperature. Sun exposure has been shown to increase phenolic and anthocyanin levels in Oregon and British Columbia, but anthocyanin levels can also decrease with high light levels (Nicholas et al. 2011). UV levels will inevitably increase with sunlight exposure, and UV radiation can have a damaging effect on vine leaves and berries. Since flavonoids act to protect the berry against UV radiation, their production may be stimulated by increased UV radiation (Song et al. 2015)

In one study (Kliewer 1970) where temperature effects were controlled for, accumulation of anthocyanin in the berry skin was maximised at relatively high light levels. High temperatures, however, inhibited the formation of anthocyanins. With

Pinot Noir, anthocyanin production was always highest for a particular temperature treatment with high light intensity.

Fruit that was highly exposed to sunlight but artificially cooled had increased anthocyanins, while conversely less exposed fruit that had been heated had decreased anthocyanins (Spayd et al 2002, cited in Nicholas et al 2011). Wines made from Pinot Noir clusters that were highly exposed to the sun had an anthocyanin concentration 60 % higher than wines made from shaded clusters, and 14 % more than wine made from moderately exposed clusters (Mazza et al. 1999).

Another study (Price et al. 1995) looked at the impact of sun exposure on Pinot Noir in the Willamette Valley, Oregon, USA. The study found that wine made from grape clusters exposed to the sun had higher levels of anthocyanins and quercetin than from shaded bunches. Interestingly, the higher anthocyanin levels were not observed in the berries themselves, and the authors attributed the difference in part to the smaller berry size (and hence greater skin:juice ratio) of sun-exposed grapes. In contrast, catechin levels (a copigment) increased in shaded berries (Price et al. 1995)

An NMR-based metabolomic study (Rochfort et al. 2010), which looked at shaded and unshaded Shiraz and Cabernet Sauvignon grown in the Sunraysia region, Victoria, Australia., In this study, it was noted that the wine from the shaded grapes was a more ruby in colour than violet. Despite the use of NMR, the study did not discuss differences in anthocyanins or other phenolics that may have accounted for the observed differences.

The impact of UV radiation and leaf removal has been studied using Pinot Noir (Song et al. 2015), in Tasmania. This found that sunlight exposure increased wine colour

density by 54 %, anthocyanins by 45 %, total pigments by 46 %, total phenolics by 63 %, and tannins by 121 %; UV exposure increased wine colour density by 79 %, anthocyanins by 106 %, total pigments by 66 %, total phenolics by 85 %, and tannins by 202 % (Song et al. 2015, p. 426). The authors noted that this was

> "*likely to be due to the increased flavonol and flavonoids due to the activation of genes of the phytopropanoid pathway in grape berries simulated by UV radiation*" (Song et al. 2015, p. 426),

This view was shared by Gregan et al. (2012).

A recent study (Del-Castillo-Alonso et al. 2016) measured the metabolite profile of Pinot Noir berry skins from winegrowing regions across Europe, from Jerez (in the south of Spain) to Geisenheim (in the middle of Germany). They reported that UV radiation seemed to have the biggest impact on phenolic levels (especially flavonols and flavanonols), and that higher UV radiation levels, as found in more southerly latitudes, produced higher levels of phenolics. Temperature was found to have a weak effect on phenolics, but only during the period from *véraison* to harvest, and this impact was not as strong as that of UV radiation. Warmer temperatures were found to produce more phenolics (including anthocyanins) than cooler temperatures. They did conclude, however, that temperature was particularly important for anthocyanins.

1.3.5 Clones

Different clones of Pinot Noir differ in grape and wine colour (Butler 1981; Robinson, J & Harding 2015); the *Oxford Companion to Wine* noted that:

> "*It is possible to choose a clone of Pinot Noir specially for the quality of its wine, its productivity, regularity of yield, resistance to rot, and/or*

> *for its likely ripeness (which can vary considerably). A major factor in the lighter colour and extract of so much red burgundy in the 1970s and 1980s was injudicious clonal selection, resulting in higher yields but much less character and concentration in the final wine*" (Robinson, J & Harding 2015, para. 3).

Certain clones are known for producing higher quality wines, others for higher yields. Pinot noir clones can be placed into a number of different categories, including '*Pinot droit*' and '*Pinot tordu*'. '*Pinot droit*', often called 'upright pinot', has shoots that grow straight upwards from its trunk; '*Pinot tordu*', also called '*Pinot Fin*' or '*Pinot Classique*', has shoots that grow at an angle into the alleys between the vine rows. '*Pinot droit*' vines are known for producing high yields of mediocre quality grapes, while '*Pinot tordu*' vines include some of the best quality selections from Burgundy. '*Pinot tordu*' vines will typically produce smaller berries with thicker skins (Haeger 2004; Robinson, J & Harding 2015).

Some examples include the "Dijon" clones from ENTAV (L'Établissement National Technique pour l'Amélioration de la Viticulture, Le Grau du Roi, Languedoc-Roussillon, France). Dijon 115, popular in North America for its "brilliance and perfume" (Haeger 2004, p. 138), was selected in France primarily as a consistent producer. Some more recent selections (with correspondingly higher numbers, e.g. 667, 777, and 828) have been noted for their

> "*impressive concentration, great intensity and depth of black fruit flavours*" (Haeger 2004, p. 138).

The *Oxford Companion to Wine* noted that clone 667 had been found by top Côte d'Or (Burgundy, France) producers to make wines that were marginally better in quality than that of 777 or 828 (Robinson, J & Harding 2015). These later clones are

amongst the earliest to ripen, however, and there is concern amongst growers that this may make them less suitable for Californian conditions, a concern that would be of equal validity in most of mainland Australia. Pommard (UCD4, 5, and 6, the latter two being heat treated variants) is of interest here because it is, amongst other things, noted for its good colour. In contrast, the so-called Gamay Beaujolais clone (UDC 18, 19, 20, 21, & 22), which is neither Gamay nor from the Beaujolais region of France, is known for high yields and large clusters. It is an example of a '*Pinot droit*' clone (Haeger 2004).

Clones usually perform true to type wherever they are planted, meaning that clones can be selected to fit desired organoleptic qualities. However, clones planted on particularly shallow or deep soil can perform differently. It is also known that there can also be regional eccentricities, e.g. the performance of UCD 2A on the southern Central Coast of California (Haeger 2004, p. 147). Of particular interest to the present study is that that several Australian researchers have found that Pinot Noir clones behave quite differently in different climates (cited in Haeger (2004, p. 147), though unfortunately not being a peer-reviewed source he does not cite his references for these claims). It is also important to note that site quality is still considered to have more impact on wine quality than clone: good clones cannot compensate for a poor site, and the potential of a great site will not be entirely obscured by a poor choice of clones (Haeger 2004, p. 148).

In an Australian context, a number of clones have been evaluated in the context of the *terroir*/environment of Tasmania. Clones 114 and 115 exhibited lower berry weight and higher colour. Clone 2340 (MV6), a popular clone in Australia, was noted as having poor colour and low aroma. Newer clones such as 667 and 777 were

not included in the trial, so trials which included these newer clones would be of value (Farquhar 2003).

1.3.6 Yield and vine spacing

Yield also cannot be disregarded. Lower yields are reported to give more intense aromas (Farquhar 2003). This view is also common in the industry. Henri Jayer, one of Burgundy's most respected *vignerons*, noted a link between yields, skin thickness and colour:

> "*Smaller yields give more complete wines, with a more complex texture, much silkier. The skins are thicker, for interesting color and aromas!*" (Rigaux 2009, p. 117).

In contrast, Heazlewood (2005) noticed that small bunches developed more intense colour independently of fruit load, and that heavier cropping vines did not inhibit colour development. As noted in the previous section (see section 1.3.5), yields and berry sizes can also differ between clones.

Vine spacing may also impact colour. One study (Hunter et al. 1996) showed that berries from closer spaced vines (1 m × 0.5 m, with the widest spacing trialled being 3 m × 3 m) produced the highest levels of anthocyanins in the skin (measured as mg/skin, methodology not explained). Additionally, these closer spaced vines had smaller berries, potentially providing higher skin:pulp ratio, leading to increased colour extraction. The study suggested plantings of 2 m × 2 m or 2 m × 1 m as optimal, balancing all factors (including non-quality related factors such as labour inputs) (Hunter et al. 1996). However, the economic considerations of course depend

also on the price at which the wine can be sold for, especially if the cost of any additional labour input can be recouped by higher prices.

1.4 Winemaking

1.4.1 Maceration

Maceration methods used are generally designed to promote the extraction of tannins and colour (Dambergs et al. 2012).

Cold maceration is a technique that is commonly used in industry in producing Pinot Noir, and is believed to improve colour extraction (Dambergs et al. 2012). Its popularity is partly due to the influence of Henri Jayer, who was a proponent of cold maceration, noting that:

> "*Maceration extracts the aromas and creates a very beautiful, enduring color*" (Rigaux 2009, p. 125).

Cold maceration involves holding the must at a low temperature, with added sulfur dioxide. Easily extracted anthocyanins are then able to enter the must, and tannin extraction is helped by the general breakdown of cellular tissue. The effect can be improved by *saignée*—bleeding off some of the juice after crushing, so that skin tissue components are concentrated (Dambergs et al. 2012).

Numerous studies have investigated the impact of various winemaking techniques on colour extraction.

Examples include a study that looked at the effects of cold maceration on wine made from Tuscan Sangiovese grapes (Parenti et al. 2004). Both solid carbon dioxide ("dry ice") and liquid nitrogen were trialled, at 5 °C (CO_2 and N_2), 0 °C (CO_2 and N_2)

and -5 °C (CO_2 only). A control batch was made without the addition of a cryogen. The rationale was that ice crystals forming within the berry skins would break cell walls, leading to the leaking of protoplasm. Both chemical and sensory analysis was undertaken, including work on colour. Colour was measured at 420, 520, and 620 nm, and a wavelength scansion between 230 and 700 nm of wine diluted with acidic ethanol solution (to determine the composition of phenolic fractions). Of the batches produced, N_2 at 5 °C and 0 °C produced the wines with the highest colour density values. Colour hue values were actually lower for those treated with N_2 than the control, in part due to the impact of oxidised components on colour hue. CO_2 at 5 °C had the least colour intensity, indicating it to be a poor quality wine, possibly due to the effect of oxidative enzymes produced by *Botrytis cinerea*; this ferment was affected more than the others due to the higher temperature at which the maceration took place. Analysis of the phenolic fraction showed that the trends in flavonoids and anthocyanidins were similar to those for total phenolic compounds. Proanthocyanidins were influenced by the cryogen used. Extraction of these compounds by CO_2(s) was lower and more temperature-independent than N_2(l). With N_2(l), extraction increased at lower temperatures.

Another study looked at the impact of addition of dry ice to cold maceration(La Forgia 2011). This study looked at Pinot Noir MV6 from Mt Barker, South Australia, Australia, and Pinot Noir 115 from Russian River, California, USA. Both traditional cold maceration and dry ice treatments were undertaken. Different forms of dry ice were used in the US and Australia, with dry ice “rice pellets” used in the US, and larger blocks of dry ice used in Australia. Analysis was undertaken using both HPLC and UV-Visible spectrophotometry (using both the Sommers and CIELab methods). Analysis of copigmentation and polymerised anthocyanins were

undertaken using a method initially described by Boulton (2001, cited in Hermoín Gutiérrez 2003), then further built upon in later papers (Hermosín Gutiérrez 2003). The study noted that wines of greater colour concentration could be made using maceration with dry ice, particularly in the presence of sulfur dioxide. Larger blocks of dry ice were apparently more effective than the "rice pellets" form.

Another study (Heatherbell et al. 1996) also looked at cold maceration in New Zealand Pinot Noir. Cold maceration was done at different temperatures (4 °C and 10 °C) and with varying degrees of sulfur dioxide (0, 50, and 100 mg L^{-1}). With this study, total phenols and colour parameters were determined by spectral methods (presumably spectrophometry). Cold maceration caused an increase in visible colour, especially of red-purple hues, in young wines. This increase correlated with similar increases in total phenols, colour density and total monomeric anthocyanins. This increased extraction only occurred in the presence of sulfur dioxide, suggesting it plays a role in the extraction of phenolics from the grapes. The study did not reveal any difference in relative percentages of anthocyanins between methods, or in hue or degree of ionisation of anthocyanins between the control and cold maceration wines. Increasing maceration temperature likewise had no impact on compositional and colour parameters, but did have an impact on visual colour, with wine macerated at 10 °C having less colour.

A further study (Parley, Vanhanen & Heatherbell 2001) looked at the impact of preferment enzyme maceration on extraction and colour stability in Pinot Noir wines. A number of different treatments were trialled: a control, cold maceration with no sulfur dioxide or enzymes, cold maceration with sulfur dioxide but no enzyme, cold maceration with VR-C enzyme but no sulfur dioxide, and cold maceration with sulphur dioxide and VR-C enzyme. At pressing, the wines that were

not treated with enzymes had relatively high levels of monomeric anthocyanins, while those that were treated with enzymes had lower levels. The wines were retested at bottling (2 months after pressing), and a total phenolic analysis showed that all the wines had very low total phenolics as measured by absorbance at 280 nm, but that preferment maceration, especially with sulfur dioxide and enzyme, produced significant increases in phenolic content. Individual phenols were separated by HPLC with peaks at 280 nm, 320 nm, and 520 nm. Again, maceration led to a noticeable increase in polymeric pigments, but the picture is not so clear with other compounds, e.g. maceration seems to have little impact on caftaric acid. The wines were then re-examined with additional ageing (discussed later in this review). Polymeric pigments were also determined by HPLC at 520 nm and eluting after the monomeric anthocyanins. The amount of polymeric pigments measured exhibited an inverse trend in relation to monomeric anthocyanins—i.e., treatments that were lower in monomeric anthocyanins were also higher in polymeric pigments, and that this became more evident at three and six months of bottle ageing (Parley, Vanhanen & Heatherbell 2001).

It is interesting that the enzyme treatments above had the greatest amount of polymeric pigments. From this, Parley, Vanhanen and Heatherbell (2001) deduced that the majority of the monomeric anthocyanins eventually end up in coloured polymers. While this will occur in all the wines, the authors of this paper considered that the presence of macerating enzymes will hasten this transformation. They remarked that this inverse relation between polymeric pigments and monomeric anthocyanins has been noted before by other authors (e.g., Wightman et al. 1997 cited in Parley, Vanhanen & Heatherbell 2001), and is recognised as a general phenomenon in red wines. Wines that were treated with sulfur dioxide during

preferment maceration were also found to have more polymeric pigments after bottling than the controls. This may have been due to the yeasts producing higher levels of acetaldehyde in response to the higher sulfur dioxide levels.

More recently, work has been done to investigate the benefits of microwave maceration for Pinot Noir wine production. Microwave maceration is a novel thermovinification method that uses microwave energy to heat the must (Carew, Sparrow, et al. 2014).

Carew, Sparrow, et al. (2014) investigated the use of microwave maceration on Tasmanian Pinot Noir wines, using 1 L scale fermentations. Must was heated to 70 °C using a domestic microwave oven, held at that temperature for ten minutes, then allowed to cool prior to inoculation. The microwave maceration wine was found to have significantly higher total phenolics and anthocyanins than the control wine. The study also found significant differences in colour density and hue between the microwave maceration wine and the control wine. These differences were still apparent after six and eighteen months of bottle ageing. The authors also stated that microwave maceration effectively sterilised the must, allowing for slightly lower sulfur dioxide usage—though at disadvantage of preventing the use of a spontaneous ferment (Carew, Sparrow, et al. 2014).

This was reinforced by Carew, Gill, et al. (2014), who also investigated the impact of microwave maceration on Tasmanian Pinot Noir wines. Their study also compared microwave maceration with conventionally heat treated must (i.e., conventional thermovinification). Their study noted that microwave maceration must was higher in yeast assimilable nitrogen than the control must or conventionally heat treated must, and had better fermentation kinetics. They also

noted that the microwave maceration wine started and finished fermentation earlier than either control or conventionally heated must, due to a shorter lag phase.

The same technique has also been found to be effective with other grape cultivars such as Shiraz. In addition, it was also found to eliminate laccase from juice, which is potentially of interest in regions prone to *Botrytis cinerea* infection (Carew et al. 2013). The disadvantage of microwave heating is that it is expensive to scale up.

1.4.2 Stems—whole bunch ferments and stem return

Many wineries that produce Pinot Noir wines include stems in the ferment, often as whole bunch ferments. Stems can be included in the ferment either by not destemming (whole bunch ferment), or by destemming and then adding stems back into the ferment (Butler 1981).

Famous examples of producers that use whole bunch ferments include Domaine Dujac and Domaine de la Romanée-Conti in Burgundy (Goode 2012; Nanson 2009). Opinion is divided amongst winemakers as to the benefits of stem inclusion, with some believing that it enhances colour (perhaps due to copigmentation, with tannins extracted from the stems), and others claiming that the stems leach colour from the wine (Goode 2012). Henri Jayer was a keen proponent of destemming, claiming that:

> *"The rafle [stem], seldom very ripe, would only bring astringent, acerbic tannins"* (Rigaux 2009, p. 124).

Boulton also thought that the addition of stems would be detrimental to wine colour, stating that:

> *"The additional color loss by adsorption onto stems when they are present has been well documented, and this appears to be an extension*

> *of the more general adsorption onto pulp and yeast"* (Boulton 2001, p. 82).

Unfortunately, results from the scientific literature are also inconclusive. Several studies have looked at the impact of stem inclusion on wine composition and quality (Butler 1981; Dambergs et al. 2012; de la Barra et al. 2005; Pascual et al. 2016). One study, which made particular reference to the impact on wine colour, examined the impact of stems on wines made from Pinot Noir grown in Mt Crawford in the Barossa Hills, South Australia (Butler 1981). This paper noted that the aim of adding stems (rachis, pedicel, and peduncle) to the ferment was to stabilise colour by increasing the phenolic content. Three treatments were trialled: a control, with no stems added back, 33 % of stems added back, and 66 % of stems added back. Both levels of stem addition led to wines that had reduced titratable acidity, as well as increased pH and potassium concentration. They also note that addition of stems has been previously shown to reduce alcohol and acid content. The pH and potassium concentration differed significantly at every level of stem addition. The changes in pH may well have been caused by the extra potassium extracted from the stems. Wine colour density ($E_{420} + E_{520}$) did tend to increase with stem addition, particularly after the addition of sulfur dioxide (though the readings initially reduced slightly due to bleaching). They note, however, that this contradicted earlier studies.

A more recent work examined a number of treatments, including return of 20 % of stems to the ferment. The grapes used were Pinot Noir, grown in the Krems Valley in Lower Austria. Here, the stem return wine was had increased colour intensity compared with the control ($A_{420} + A_{520}$, determined by spectrometry), but less so than the other methods trialled (re-heating the must, to 45 °C for 42 hours, using a

heat exchanger), stem return and re-heating combined, and addition of mannoproteins) (de la Barra et al. 2005).

Another trial (Dambergs et al. 2012) concerned the impact of stem inclusion on Tasmanian Pinot Noir wines, amongst other factors, by adding back 30 g kg^{-1} (i.e. 3%) of stems. Trial ferments were microvinified in Bodum 'French Press' coffee plungers with 1 kg of grapes, using RC212 yeast. All ferments had 50 mg kg^{-1} sulfur dioxide and 200 mg kg^{-1} diammonium phosphate added. The wines that had stems added back in were high in tannin, albeit not in pigmented tannin.

1.4.3 Yeast

The choice of yeast also appears to have an impact on colour in Pinot Noir wines.

One study found (Mazza et al. 1999) that Wädenswil 27 variety yeast gave lower phenolic content and colour density than the other seven yeasts trialled with Pinot Noir. It was hypothesised that this could be due to the slower rate of fermentation provided by this yeast, but no evidence was provided to corroborate this. Interestingly, Brouillard, Chassaing and Fougerousse (2003) state that copigments are frequently found in yeasts.

In contrast, Boulton (2001) notes that yeast can cause colour loss via adsorption. This point made by Boulton (2001) was reinforced by Vasserot, Caillet and Maujean (1997), who studied the potential for using yeast lees recovered from Chardonnay ferments to remove excess colour from Pinot Noir and Pinot Meunier base wines destined for white sparkling wines. The article noted that this practice was marginal but traditional in the Champagne region of France, and suggested the technique as less damaging to wine character than use of charcoal. The study used lees from a Chardonnay ferment conducted using *Saccharomyces cerevisiae* DB10,

which were washed in water to remove residual alcohol and potassium bitartrate, and dried to avoid any organoleptic impact. This study found that the yeast lees eliminated anthocyanins by adsorption, and to a lesser extent by an enzymatic mechanism involving β-glucosidase (although this proceeded very slowly). They noted that the amount of anthocyanins adsorbed was not primarily limited by the adsorbent capacity of the lees, but by reaching a partition equilibrium that depended on factors such as temperature, adsorbent concentration, and the initial concentration of anthocyanins; the study also found charcoal to be more efficient at removing colour. Polar anthocyanins were adsorbed preferentially.

Morata et al. (2003) also looked at adsorption of anthocyanins by yeast cell walls, to see both whether there were differences in the levels of anthocyanins absorbed between yeast strains, and whether specific anthocyanins were retained to differing degrees. Ten different yeast strains were trialled, all strains of *S. cerevisiae*. These were isolated from wine regions in Spain, including La Rioja (4CV, 5CV, and 9CV), Ribera del Duero (3VA, 7VA, and 1VA), and Navarra (2EV, 7EV, and 1EV). The trial wines were made using Cabernet Sauvignon grapes. Analysis of anthocyanins was performed by HPLC-DAD. The study found differences between the degree to which anthocyanins were absorbed, with malvidin-3-glucoside and acylated derivatives absorbed at the highest rate. Peonidin-3-glucoside, petunidin-3-glucoside, and delphinidin-3-glucose were also absorbed, as were their acylated derivatives. Cyanidin-3-glucoside and its derivatives did not seem to be absorbed, which might have been partly due to its low initial concentration in the wine, or because it is the starting point for the production of the other anthocyanins through the action of methyl-transferase and flavonyl-3-hydroxylase. Additionally, it is highly hydrophilic, meaning it is more likely to stay in solution (Morata et al. 2003, pp.

4084-4085). Differences were also noted between yeast strains, with some strains (including 3VA and S6U) causing low anthocyanin absorption, while others (including 2EV and 1EV) had high absorption—for example, S6U absorbed 1.68 mg of malvidin-3-(6-*p*-coumaryl)-glucoside, while 2EV absorbed 4.13 mg. Impact on wine colour was also noted. Cell wall absorbates had lower colour intensity than the wine, mainly due to lower absorption of blue and higher absorption of yellow tones (Morata et al. 2003).

Mazauric and Salmon (2005) analysed the phenolic compounds in wine and yeast lees after ferment. This study used Cabernet Sauvignon grown near Arzens, southern France that had been fermented using *S. cerevisiae* strain K1. Analysis was undertaken using both spectrophotometer (at 280, 320, 420, 520, and 620 nm) and HPLC-DAD. Analysis showed that the lees absorbed a large proportion of coloured polyphenols (Mazauric & Salmon 2005, p. 5649). In particular, wine colour intensity was strongly affected by lees contact, while there was almost no effect on wine tint. After a week of lees contact, the wine had lost one third of free anthocyanins, whereas wine in the blank (control) experiment (presumably aged without lees contact) had lost none. The control experiment also showed no reactions between polyphenols and anthocyanins (such as copigmentation). This could have been due to the anaerobic conditions, which meant phenolic polymerisation reactions would likely not have included acetaldehyde. Acetaldehyde is thought to be a key component in oxidative polymerisation. In this study, all free anthocyanins were affected, especially malvidin-3-glucoside. Interestingly, the paper noted that yeast lees can also bind other polyphenolics, including tannins. After a week on lees in the

absence of oxygen, up to 70 % of condensed tannins had been lost (Mazauric & Salmon 2005).

Medina et al. (2005) looked at yeast interactions with anthocyanins during the fermentation of red wines. This study used a model red grape juice that was made from sterilised Chardonnay juice supplemented with anthocyanins extracted from Tannat grown in Uruguay. Use of a model grape juice prevented complications such as absorption of anthocyanins by the grape skins. A number of yeast strains were examined including two commercial yeasts (Montrachet 552 and Lalvin 254 D), as well as a number of yeasts isolated from wineries in Uruguay (*Saccharomyces cerevisiae* strains CP881, CP882, CP863, CP874, CP873, and KU1). Fermentations took place in sterilised 125 mL Erlenmeyer flasks filled to 50 mm with RGJ medium and stoppered using Müller valves, and were incubated at 20 °C with shaking. Colour intensity (A420 + A520 + A620) and hue (A420/A520) were then measured by spectrophotometer, total anthocyanins were measured at 540 nm, then identified and quantified using HPLC-DAD. Quantification was performed at 530 nm, and expressed as a function of malvidin-3-glucoside concentration. Anthocyanin derived pigments were detected by HPLC-MS.

This study showed no significant differences between strains after fermentation, with all strains trialled showing an average 17 % decrease in anthocyanin levels compared with the unfermented model juice. There were, however, significant differences between the treatments with regard to the concentrations of individual anthocyanins. All trials showed that the percentage of anthocyanin removal increased with increasing polarity. It is suggested that this is due to adsorption by yeast cells, especially given the affinity of polar anthocyanins to yeast cell walls. However, while total anthocyanins decreased in all treatments, some

maintained or increased colour intensity. The authors hypothesised that this might be due to copigmentation or the presence of anthocyanin derivatives. It was suggested that the presence of anthocyanin derivatives is most likely as copigmentation, which would be avoided under the experimental conditions. The difference between total pigment concentration and colour intensity for these strains was significant, suggesting that anthocyanin derivatives play an important role in colour, and that their formation differs significantly between yeast strains. The study noted that these results would likely be different for other cultivars, with further experiments needed (Medina et al. 2005).

A number of studies have compared the impact of various strains of *S. cerevisiae* and *S. bayanus* (Blazquez Rojas, Smith & Bartowsky 2012; Carew, Dambergs & Curtin 2012; Hayasaka et al. 2007; Takush & Osborne 2012). One paper (Blazquez Rojas, Smith & Bartowsky 2012) looked at seven different strains of *S. cerevisiae* (AWRI 796, AWRI 838, AWRI 1486, AWRI 1553, AWRI 1554, and AWRI 1555), two of *S. bayanus* (AWRI 1176 and AWRI 1375) and two interspecific *Saccharomyces* hybrids (*S. cerevisiae* × *S. paradoxus* AWRI 1501 and *S. cerevisiae* × *S. kudriavzevii* AWRI1503). Ferments were trialled using Cabernet Sauvignon grapes from Clare Valley, South Australia. Colour density was measured by spectrophotometer and a modified version of Somer's assay. Anthocyanins and pigmented polymers were identified via HPLC-DAD. With this study, it was noted that *S. cerevisiae* produced wines with lower colour density than *S. bayanus*. A hybrid strain produced the highest colour density. *S. bayanus* wines had the lowest total anthocyanins, and the highest sulfur dioxide resistant pigments. *S. cerevisiae* and the hybrids had a range of these pigment components. Both *S. bayanus* strains had high levels of pigmented

polymers, but the highest levels were found in ferments using *S. cerevisiae* AWRI 1486. *S. bayanus* is known to produce wines with higher glycerol and lower ethanol levels than *S. cerevisiae*, and high concentrations of compounds that add to wine colour density. The authors of this paper stated that it

> "*highlighted the importance of yeast strain in wine colour and phenolic composition*" (Blazquez Rojas, Smith & Bartowsky 2012, p. 3319).

They noted that the eleven different strains of yeast had resulted in a broad range of total anthocyanins, colour densities, pigmented polymers, tannin concentrations, and sulfur dioxide resistant pigments (Blazquez Rojas, Smith & Bartowsky 2012).

Another study undertaken the same year (Takush & Osborne 2012) reported differences in ferments between the yeasts trialled (*Saccharomyces cerevisiae* RC212, Assmanshausen (AMH), MERIT.ferm, *S. bayanus* EC1118, and a blend of *Kluyveromyces thermotolerans* (SYMPHONY) & *S. cerevisiae* MERIT.ferm), especially with regard to aroma. With particular regard to colour, *S. bayanus* EC1118 had higher colour at 520 nm than AMH, though no differences were noted between the other yeasts used (Takush & Osborne 2012). These differences could have been caused by acetaldehyde, since some strains of *S. bayanus* are known to produce more acetaldehyde than *S. cerevisiae* (Hayasaka et al. 2007; Takush & Osborne 2012).

Hayasaka et al. (2007) also looked at the differing impacts of *S. bayanus* and *S. cerevisiae* on Cabernet Sauvignon grapes. The study used grapes sourced from Padthaway, South Australia. A crucial distinction was made between the levels of acetaldehyde that the yeasts produced. Colour was measured using spectrophotometry, using wavelengths from 400 nm to 700 nm for absorbance. *S. bayanus* produced noticeably more acetaldehyde than *S. cerevisisae*. Colour was nearly identical 8 days after inoculation, but differences were noticeable 387 days (1

year, 3 weeks, and 1 day) after inoculation. At this point, *S. bayanus* had noticeably higher wine colour density (the sum of absorbances at 420 and 520 nm), the CIELab colour parameters, as described in Chapter 2 (section 2.3.3), of a* (redness), b* (yellowness), and L* (lightness), as well as sulfur dioxide non-bleachable pigments, pigmented polymers, and acetaldehyde. The *S. cerevisiae* had more monomeric anthocyanins, and higher L*. This was linked to levels of pyruvic acid, acetaldehyde, acetone, 4-vinylphenol, 4-vinylguaiacol, and vinyl(epi)catechin. The paper noted that it was already known that addition of acetaldehyde could enhance wine colour intensity, and the authors also reported that the characteristic enhancements of the *S. bayanus* ferments were similar to those found where acetaldehyde had been experimentally added to the wine. The lower level of free anthocyanins found in the *S. bayanus* wine was compensated by the higher level of acetaldehyde-mediated pigments. This suggested that the difference in levels of acetaldehyde production was the main difference between the two species.

Carew, Dambergs and Curtin (2012) investigated the importance of yeast for phenolics in Pinot Noir, noting that extraction and stabilisation of phenolics are particularly challenging for Pinot Noir winemaking. Their study looked at the impact of fermenting several different yeasts on Tasmanian Pinot Noir wine colour. The yeasts trialled were *Saccharomyces cerevisiae* (strains RC212, AWRI7196, and EC1118), *S. bayanus* AWRI 1176, *S. cerevisiae* × *S. Kudriavzevii* AWRI1503, *Torulaspora delbruekii* (to which EC1118 was added after four days), and a 'wild' ferment to which EC1118 was added after four days. It is worth noting that EC1118 is now considered to be a strain of *S. bayanus*, not *S. cerevisiae*. The wines were produced using small scale 1 L ferments. Tannin and pigment effects were evaluated using UV spectrophotometry. They noted that yeast treatment had a significant

impact on phenolics, with the selected *Saccharomyces* yeasts mostly producing a more stable colour and higher levels of tannins than the wild ferments, though from the data supplied the distinctions seem less clear-cut than the conclusions stated would justify. It is also likely that the simulated wild ferment was too simplistic as a simulation to emulate the effect of a real wild ferment, which may contain dozens of different yeast species at the start of the ferment (Lopandic et al. 2008; Zhang et al. 2010). Additionally, it is also possible that since the microbial flora found in wild ferments will differ from one region to the next, the impact will be very site dependant (Lopandic et al. 2008)—although Zhang et al. (2010) seem to disagree, stating that the yeasts found in grape juices in New Zealand were similar to those found in other parts of the world.

1.5 Impact of ageing on wine colour

1.5.1 Ageing in barrel

Colour changes in Pinot Noir wine during barrel ageing may also be significant. In conversation with Ross McDonald of Macquariedale Organic Wines (R McDonald, personal communication, 28th December 2012) in the Hunter Valley, he noted that his 2012 Pinot Noir had noticeably *darkened* in colour after nine months in barrel. Since this winery pursues a low-sulfur regime, it is unlikely that this was simply the result of sulfur dioxide bleaching the wine. Interestingly, Pérez-Prieto et al. (2003) noted that while colour loss from chemical changes to free anthocyanins occurs during barrel ageing, other reactions can lead to the formation of new colours. Most notably, in oxidative conditions, ethanal bridge condensation reactions between anthocyanins and tannins can produce condensed pigments that have a violet hue.

Most studies involving small scale ferments rely on fermentations conducted in glass vessels, and simulation of the commercial technique of barrel ageing is difficult. Nonetheless, Parley, Vanhanen and Heatherbell (2001) suggested that colour would probably be augmented and stabilised by exposure to oak barrels or chips. A paper by Dambergs and coworkers found that adding oak powder during ferment caused a reduction in tannin levels. The authors suggested that this may have been caused by tannin binding effects of oak, which could predominate in situations where there is a high oak particle surface area to volume ratio, and they noted that this particle size effect may require further investigation (Dambergs et al. 2012). The authors also went on to note that while ageing wine in oak barrels promotes pigment stabilisation by slow oxygen uptake,

> "*putting oak in wine (as opposed to wine in oak) is a different scenario*" (Dambergs et al. 2012, p. 25).

Old and new barrels have different impacts on the wine, partly because with prolonged use wood pores in the barrels become blocked, causing a decrease in the oxidation effect noted in barrels. After three to five uses, wines stored in barrel will have a similar level of dissolved oxygen to those stored in tanks, causing different colour characteristics to be obtained (Gómez-Plaza et al. 2004).

Barrel size is also important, as this influences the surface:volume ratio of wine that is in contact with oak. Barrels with a volume of approximately 220 L (referred to as *barriques*; with Bordeaux-style *barriques* containing 225 L, and Burgundy-style *barriques* 228 L) are often considered to be ideal, perhaps because the surface:volume ratio is particularly favourable for phenolic polymerisation (Gómez-Plaza et al. 2004).

An earlier paper by Heatherbell et al. (1996) also noted that the extra colour and anthocyanins extracted by cold maceration in unoaked wines were lost after two to three years of bottle age (i.e., there was no observable difference between the control and the cold maceration wines), but this colour loss was not observed in commercial trial wines that had been transferred to oak barrels within one week of completing fermentation. They suggest that this treatment resulted in stabilisation and augmentation of observable colour, and suggested this might arise from copigmentation with phenolics extracted from the oak. They noted the same phenomenon when oak chips were added to cold macerated wines microvinifed in glass. These wines, however, were also treated in a more oxidative manner than the others, as well as being put through malolactic fermentation (Heatherbell et al. 1996).

Boulton (2001), however, was more sceptical:

> "*There has been some suggestion that components from new oak barrels can lead to color enhancement, but it is not clear if this is simply due to adsorption of free sulfur dioxide onto freshly charred surfaces or to some extraction of components that are acting as copigmentation cofactors and complimentary to those already present in the wine.*" Boulton (2001, p. 71)

Boulton (2001) stated that instead of than enhancing colour, the surface of new barrels may absorb free pigments (anthocyanins) and cofactors. Any copigmentation effect would need to overcome this absorption to show an enhancement of colour. Boulton also noted that this effect was wine-specific in its extent, and would alter the equilibrium in effect during subsequent polymerisation reactions that occur as part of the process of ageing.

Boulton (2001) went on to suggest that the idea that barrels contribute colour via copigmentation might partly be due to the use of the sum of absorbance at 420 nm and 520 nm to measure colour intensity. Based on this measurement, any compound which led to an increase in "brownness" could be considered to increase the colour. Boulton suggested that measuring copigmentation (instead of colour density) in a young wine or model wine should be considered. This could be done after tannin fractions and extracts from oak and grape seed had been added. This, he hoped, would clarify the impact that barrels had on colour.

Gómez-Plaza et al. (2004) reported that monomeric anthocyanins decreased during both bottle and oak ageing. Their paper looked at wine produced from Monastrell (i.e. Mourvèdre) grapes in the Jumilla region of Spain. Wines were aged in new and old (third use) French (220 L) and American (220 L, 500 L & 1000 L) oak barrels for six months, then aged in bottle for a year. Colour was measured using spectrophotometry, monomeric anthocyanins were assayed by HPLC-DAD. The rate of decrease was found to be highest in oak, with the age of the barrel having more impact than the origin of the oak (French oak *versus* American oak). Barrels that had been used three times previously produced a lower decrease in monomeric anthocyanins. This decrease was not reflected in the colour density, which did not decrease by much, suggesting that the condensation products between tannins and anthocyanins are mostly responsible for colour density in aged wines. After one year in bottle, wine matured in French oak had the highest colour density. Monomeric anthocyanins decreased most in new barrels, colour density in old barrels (Gómez-Plaza et al. 2004)

Anthocyanins can react with yeast metabolites (e.g., pyruvic acid and acetaldehyde) and oak-derived compounds (e.g. fufural and hydroxymethyl furfural) to produce new pigments. Ethanol may also be oxidised to acetaldehyde, adding to the potential sources of acetaldehyde. One study used LC-MS over the course of ten weeks to track the rate at which anthocyanins disappeared. It was found that the presence of tannins or acetaldehyde increased the kinetic rate of reaction, i.e. that copigmentation reactions occurred more rapidly (Saucier et al. 2004).

1.5.2 Ageing in bottle

In a report by Brouillard, Chassaing and Fougerousse (2003), Brouillard recounted that as a young adult, he had been present when an uncle discovered a number of bottles of red Burgundy (including Romanée-Conti and Pommard) hidden under a coal heap in a Parisian cellar. This discovery occurred in the 1970's; the wines were from the 1920's and 30's, and had perhaps been hidden during World War II. The wines had largely retained their original colour.

Initially it would appear unlikely that wine pigment could be preserved over such a long period, given the fact that the anthocyanins found in grapes and young wines are much simpler than those found in other flowering plants (e.g., *Pharbitis nil*, *Petunia hybrid*, and other examples). In particular, Brouillard, Chassaing and Fougerousse (2003) pointed out that simple anthocyanins are not thought to remain stable in an aqueous medium (such as wine). For a red wine to maintain its colour, the anthocyanins must condense with tannins to form wine pigments. One such group of wine pigments known to have a role in aged wines are the Vitisins. In addition, a phloroglucinol A-ring is essential in the formation of red wine pigments, as is flavylium-5-OH (Brouillard, Chassaing & Fougerousse 2003). This point is

reiterated by Revilla, I et al. (1999), who attributed the disappearance of free anthocyanins and the shift from red-blue to orange brown colour during wine ageing to the replacement of unstable free anthocyanins by stable polymeric coloured compounds. These chemical changes thus allow the wines to retain their colour.

The impact of ageing on wines that had undergone cold maceration has also been examined (Parley, Vanhanen & Heatherbell 2001). The wines were analysed at three, six, nine, and eighteen months after bottling. At eighteen months of age, the control had the highest levels of monomeric anthocyanins, although levels had declined in all wines. With regards to polymeric pigments, the authors noted that these increased up to three months in bottle, but from that point until eighteen months of bottle age they declined to a minimally detectable level. This was considered likely to be caused by precipitation of larger molecule aggregates from solution. The authors note that the sulfur dioxide and enzyme treated wines had higher amounts of polymeric pigments compared to the controls, and that a decrease in colour density did not accompany a loss of polymers—instead, colour actually *increased* in all but the control wine, indicating that some degree of colour stability had been conferred by the remaining polymers.

Maceration treatments with sulfur dioxide, with sulfur dioxide and enzyme, and with enzyme alone all increased visible colour, the sulfur dioxide/enzyme treatment producing the most intense colour. At three months after bottling, the enzyme treated wines had greater perceived colour intensity, and a more red-purple hue. After six months in bottle, this visual difference was even more noticeable. Increases in perceived visual colour were found to correspond to increases in measured colour density. This increase in colour density may have been due to the observed increase

in the formation of polymeric pigments, as the wines with the greatest colour density were consistently those with the highest polymeric pigment contents. The enzyme treatment therefore appeared to produce the most coloured wines from bottling onwards, by promoting the increased formation of stable polymeric pigments. The authors also noted a trend for wines that were treated with sulfur dioxide prior to ferment, with or without enzymes, to produce wines with a lower hue—i.e., more redness and less browning—but this trend was not statistically significant (Parley, Vanhanen & Heatherbell 2001).

The same paper (Parley, Vanhanen & Heatherbell 2001) also discussed the concept of "chemical age". The chemical age index is determined from spectral measurements at 520 nm and expresses the proportion of total wine colour due to polymers, so that wines with higher polymeric pigment levels and lower monomeric anthocyanins levels would have a greater 'chemical age'. This method has the weakness that the contribution of polymeric pigments can be over-estimated due to bleaching by the sulfur dioxide used in the method. Nonetheless, the 'chemical age' results were consistent with HPLC analysis which showed an increasing ratio of polymeric pigments to monomeric anthocyanins. However, where HPLC results show a loss of polymeric pigments after six months of bottle ageing, the chemical age index did not stop increasing. Even with this loss, it showed that polymeric pigments still constituted an increasing proportion of total wine colour, and could be increasing in resistance to sulfur dioxide bleaching, or increasing in colour intensity, despite monomeric anthocyanins continuing to decrease. Alternatively, the authors considered that this decline noted by HPLC could have been caused by larger coloured polymers forming over time but not being eluted from the HPLC column by the gradient used (Parley, Vanhanen & Heatherbell 2001).

1.6 Copigmentation

1.6.1 Definition

Boulton (2001) defined copigmentation as

> "*A solution phenomenon in which pigments and other noncolored organic components form molecular associations or complexes. It generally results in an enhancement in the absorbance and in some cases, a shift in the wavelength of the maximum absorbance of the pigment*" (Boulton 2001).

Equally, Brouillard and Dangles (1994) defined copigmentation as

> "*the hydrophobic association of an anthocyanin chromophore with the planar electrically unsaturated part of a copigment*" (Brouillard & Dangles 1994, p. 366)

This association between pigments and copigments (or copigmentation cofactors) involves the anthocyanin glucosides, and particular flavonoids, tannins, flavonol derivatives, and flavones subgroup derivatives (Boulton 2001).

Boulton (2001) described the copigmentation effect as a dynamic equilibrium, and suggested it is of central importance to understanding the limitations of anthocyanins capture, colour potential, and pigment retention. Only 30—40 % of pigments are extracted from the grape skins prior to, during, or after the ferment. There is an equilibrium established based on adsorption-desorption between the concentrations in the wine and in grape skin cells, which increases significantly as alcohol levels increase. Copigmentation shifts pigments from the free anthocyanins pool of this equilibrium, allowing more anthocyanins to move from the skin to the wine. The limiting factor, therefore, is the concentration of these particular cofactors

in the skins at harvest, and their solubility in wine. Because of this, Boulton stated that additional skin contact could not provide additional wine colour or anthocyanins content. Extended contact can provide additional tannins, but Boulton dismissed the possibility that this alone could lead to further capture of pigments. (As an aside, it is worth noting that the winemaker may decide that the additional tannins are desirable for the particular wine style being produced, as is traditional with styles such as Barolo).

1.6.2 Chemistry of copigmentation

Copigmentation can be divided into two types: intramolecular and intermolecular (Boulton 2001; Brouillard, Chassaing & Fougerousse 2003). Brouillard, Chassaing and Fougerousse (2003) noted that the intramolecular copigment effect is more efficient protection against nucleophilic attack by water, leading to greater colour loss than the intermolecular copigment effect. They also pointed out that full colour stabilisation (albeit with some variation in colour) is best achieved if the anthocyanins have more than one aromatic acyl residue from the cinnamoyl acid family. The example provided is a series of anthocyanins extracted from the flowers of *Pharbitis nil* (commonly known as “Morning glory”), where approximately 94 % of the pigment chromophore is protected against hydration—e.g. see Figure 1.2.

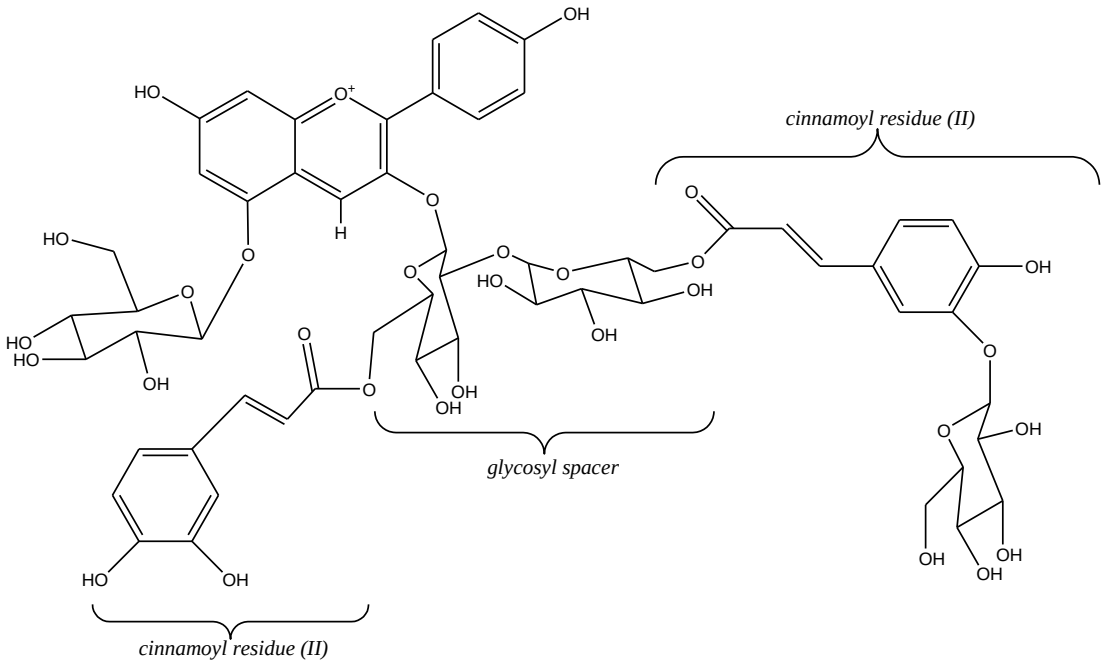


Figure 1.2: 3-O-(2-O-(6-O-(trans-3-O-(β-D-glucopyranosyl)caffeyl)-β-D-glucopyranosyl)-6-O-(trans-caffeyl)-β-D-glucopyranosyl)-5-O-β-D-glucopyranosyl pelargonidin, from *Pharbitis nil* ("Morning glory") - after Brouillard, Chassaing and Fougerousse (2003).

There is still only limited understanding of the physical properties of copigmentation associations in solution, it is presently thought that it is mainly due to hydrophobic and π- π interactions leading to planar stacks (Boulton 2001; Brouillard, Chassaing & Fougerousse 2003). Both Boulton (2001) and Brouillard, Chassaing and Fougerousse (2003) referenced the role of hydrogen bonding, but Boulton (2001) suggested that this hypothesis is no longer widely accepted. This has been determined via circular dichromism and hydrogen NMR spectroscopy. Whilst stoichiometry suggests that most copigmentation aggregates seem to be approximately a 1:1 ratio of anthocyanin to cofactor, it is to be expected that a distribution of stacks from two to ten sheets is possible. Copigmentation is a different phenomenon from the formation of pigment metal complexes, and it is unlikely that metal complexes occur in *V. vinifera* wines (Boulton 2001).

Copigmentation pushes the wine from a red colouration towards blue. The potential copigment include tannins, ethyl gallate, and aesculin. Gentisic and protocatechuic acids, quercetin, and vanillin have moderate effects. Arburtin, tyrosine, *p*-hydroxybenzoic, and salicylic acid have very weak effects. Gallic acid has some effect, but quercetin, 3-glucoside, and chlorogenic acid have no impact on the colour spectrum of cyanidin 3-glucoside (Boulton 2001).

Brouillard and Dangles (1994) discussed the role of copigmentation in wine ageing, noting that copigmentation could explain the changes in anthocyanin reactivity and wine colour seen in ageing red wines. Copigmentation can be detected easily using visible absorption spectroscopy; concentrated solutions (e.g. 10^{-3} M) can be analysed using NMR. For copigmentation to be measurable, though, anthocyanins must be present at a level that can be detected, and the copigment to pigment ratio needs to be fairly high, though this depends on the exact copigment—a higher ratio is needed if the copigment factor is weak. Strong copigments, such as rutin, can react at approx. 1:1 ratio, but more typically the range is between 10:1 and 100:1. One molecule of copigment forms a noncovalent stack (bound by Van de Waals interactions or solvent effects) with one molecule of anthocyanin in the quinoidal base or flavylium form. The copigment causes water molecules to be expelled from the pigment solvation shells into the bulk water. This provides protection from nucleophilic attack by water for the flavylium chromophore, which can lead to colour loss (Brouillard & Dangles 1994, p. 366). Copigmentation will also increase the concentration of coloured forms of anthocyanins, as copigments do not associate with inactive, non-coloured anthocyanins. Consequently, the reversible hydration process that forms inactive anthocyanins tends to be inverted to favour active anthocyanins (Brouillard & Dangles 1994).

The structural features required of a copigment are still uncertain. Those tested to date are mostly flavonoids and purine bases. It is suggested that copigments would need a planar π-electron rich ring, so that a large π- π overlap can occur with quinoidal bases and flavylium ions, forming vertical stacks that can be detected by NMR spectroscopy (Brouillard & Dangles 1994, p. 367).

Both pH and temperature are also important factors influencing copigmentation. With regard to pH, copigmentation is most effective within the pH range of 3 to 5. The exact optimal pH for colour augmentation is different for each anthocyanin and copigment combination—e.g. malvin copigmented by chlorogenic acid has an optimum pH of about 3.6. Since for many anthocyanin/copigment combinations, the optimum pH falls within the wine pH range, it is expected that copigmentation is a general phenomenon across all red wines. pH effects are mainly related to anthocyanin structure, and do not exert any measurable changes to the structure of the copigments. Brouillard and Dangles (1994, pp. 367-368) suggested that neither electrostatic repulsion nor attraction nor hydrogen bonding play a strong role in copigmentation. While its importance is often overlooked, the authors argued that temperature may be an important factor for copigmentation, noting that temperature changes can have a strong impact on the visible spectrum of a copigmented solution—the example given was the absorption band for malvin in the presence of (+)-catechin at pH 3.5, which varies with temperature but does not do so significantly for malvin in the absence of a copigment. This led them to suggest that temperature increases can bring about a partial dissociation between the anthocyanin and copigment, leaving the anthocyanin vulnerable to covalent hydration and fading. Additionally, the chemical structure of liquid water acts in

favour of complex formation in the form of hydrophobic interactions. Increasing the temperature causes partial disruption of liquid water structure, leading to the number of hydrogen bonds per water molecule being reduced. The authors also noted that the impact of temperature on the maturation and ageing of red wines is undisputed (Brouillard & Dangles 1994, pp. 369-370).

1.6.3 Tannins and copigmentation

Boulton (2001) stated that while some researchers and winemakers believe that tannins are responsible for complexing with anthocyanins to form deeper colours, this has yet to be conclusively proved. Early studies looking at copigmentation found that adding tannin modified the colour of malvidin 3-glucoside solutions towards blue, rather than red (Willstätter, Richard & Zollinger 1915, cited in Boulton 2001; Willstätter, R. & Zollinger 1917, cited in Boulton 2001). However, Boulton (2001) considered that while the term "tannin" was used in these papers, it

> "*may have been a crude extract that contained significant levels of monomeric cofactors and dimers*" (Boulton 2001, p. 71).

Ribéreau-Gayon (1974) added tannin from pine bark to an anthocyanin solution, a solution designed to simulate red wine, and noted an increase in colour intensity. Boulton (2001), however, attributed this increase to the carryover in absorbance at 520 nm from the brown colour of the tannin preparation itself, and suggested that there was no evidence of colour enhancement. In contrast, the addition of grape seed tannin to a solution of 200 μM malvidin 3,5-diglucoside at pH 3.2 had no significant impact on colour. Addition of the flavonol glycoside rutin (quercetin-3-rhamnoglucoside) did impact colour, shifting absorptions maxima toward the blue region (Boulton 2001; Scheffeldt & Hrazdina 1978).

A large study by Kambeck (2004) compared the impact of pigmented tannins (Nutrinova, GrapEX, Christian Hansen, Erelte 2000, and Overseal) and non-pigmented tannins (white grape extract, GrapeSEEDEx, and VRSupra) on the colour of Riverland Shiraz. Of the tannins used, GrapeseedEx, Nutrinova, and GrapEX had the lowest levels of polymeric pigments (71, 72, and 70 % respectively), compared with white marc extract, Overseal, Erelte 2000, and VRSupra (85, 84, 83, and 81 % respectively). Tannin additions were performed at crushing and with finished wines. Measurements, using a spectrophotometer with the wines adjusted to a pH of 3.5, were made of colour density (defined as $A_{420} + A_{520} + A_{620}$), wine colour hue ($A_{420}/A_{520}$), %Degree of red pigment colouration ($A_{520}/A_{520}^{HCl} \times 100$), sulfur dioxide resistant pigments (A_{520}^{SO2}), total red pigments (A_{520}^{HCl}), and total phenolics (A_{480}^{HCl}-4) (Kambeck 2004).

Where tannin was added at crush, Kambeck (2004) found that the colour density (with the exception of Nutrinova and GrapEX tannins), and colour hue were not significantly impacted by addition, compared with the control. An increase in colour density was noted for all samples taken after racking, as opposed to those taken after pressing. Sulfur dioxide resistant pigments were slightly higher following Nutrinova tannin addition after pressing, but no others showed any significant differences. Pigment colour was significantly higher following addition of Christian Hansen and VSupra tannins, but not for any other treatments. Total red pigments were significantly decreased in the presence of GrapeseedEx and Christian Hansen tannins after the first racking. Pigments were significantly increased by GrapEX and decreased by Christian Hansen tannins in finished wines. With total phenolics, the phenolic material was increased with Overseal tannin but decreased with

GrapeseedEx and GrapEX after racking. Nutrinova and Overseal tannins both showed increased concentration of phenolic material in the finished wine (Kambeck 2004).

The same tests were conducted on wines where the tannin extracts had been added to the finished wine. With this treatment, all tannin extracts provided an increase in colour density with comparison to the control wine, with Christian Hansen tannin providing the greatest increase. However, all wines were also shown to decline in colour hue expression. The percentage degree of red pigment colouration also increased for all of the trial wines, as did the concentration of sulfur dioxide resistant pigments, total red pigments, and total phenolics (Kambeck 2004).

Boulton (2001) provided a note of scepticism, noting that

> "*the attainment of pigment and color maxima [occurs] during the first half of traditional fermentations (punched down or pumped over) and that further skin contact has no effect in increasing pigment content or retention*" (Boulton 2001, p. 81).

He stated that the use of alternative regimes—e.g. pre-soak, post-ferment extended maceration, continuous pumpovers, use of a submerged skin cap, punching down of the cap, complete or fractional drawing of the juice from the skins prior to return (*de l'estage*) or rotary fermenters—have yet to demonstrate any ability to modify the pigment equilibrium (between pigments and copigments—see above section on copigmentation), other than by temperature effects, most of which seem to be short-term in nature.

Boulton (2001) appeared to be sceptical of the role played by tannins in copigmentation, stating that

> "*It is important to note that virtually all studies of copigmentation phenomena have focused on the monomeric components as cofactors and that there does not appear to be any evidence in model solutions of polymeric phenols (tannins) being copigmentation cofactors*" (Boulton 2001, p. 71).

On the other hand, Saucier et al. (2004) found evidence of tannins forming copigmentation complexes with anthocyanins. Their study, however, was conducted in model wine, and therefore may not reflect how copigmentation occurs in a real wine.

1.7 Summary

As seen from reviewing the literature, numerous factors can influence Pinot Noir wine colour and character; the interactions between these factors are critical but complex. Viticulture is particularly important, while climate, soil, yeast clone, and light intensity can also be important. Winemaking and ageing are also critical, with some techniques either increasing or reducing wine colour intensity.

Regionality is key for Pinot Noir wine character. Reports such as those of Roullier-Gall, Lucio, et al. (2014), Roullier-Gall, Boutegrabet, et al. (2014), and Barnard (2016) have all discussed the impact of *terroir* on Pinot Noir wines. The importance of regionality has also been shown by metabolomics-based studies such as Brescia et al. (2002), who have demonstrated that regionality can be described based on the metabolome of wines. Little in the way of similar work has yet been undertaken in Australia. Both NMR and ICP-MS data are presented in Chapter 4, with the overall aim of beginning to investigate regionality in Australian Pinot Noir wines.

Climate is perhaps one of the key factors. Studies such as those of Kliewer (1970) and Yamane et al. (2006) have shown that climate can impact colour; this work was followed up by Nicholas et al. (2011), who found similar results for intra-vineyard climatic variation. The effect of climate will be examined in greater detail in Chapter 2.

Given the complexity of the issues, this is an area with many potential research questions. This thesis aims to address a few of the questions posed, but much more work remains to be done.

1.8 Aims and hypotheses

The project was divided into three separate sub-projects:

(a) One study aimed to investigate the impact of climate on colour in *Vitis vinifera* L. 'Pinot Noir' grapes and wine, using the *in situ* passive heating system devised by Sadras and Morán (2012). It was hypothesised that increased temperatures would decrease the colour of the resultant grapes and wine. This is presented here as Chapter 2.

(b) The other study looked at several factors impacting the fermentation of Pinot Noir wines. These are presented as Chapter 3, and are divided into two minor projects:

   a. To test the use of commercial coffee pots with plungers as a means of conducting small scale fermentations under controlled and reproducible conditions (Dambergs et al. 2012; Liccioli pers. comm., 2014), and to assess if location on the vineyard slope (which was a

potential confounding factor for the heating treatment trials, Chapter 2) had any impact on wine colour in isolation from the other studies. It was hypothesised that the 'Bodum' coffee pots will be effective as fermentation vessels, and that the location on the slope will not affect wine character.

b. To test whether the inclusion of stems in fermentations would impact colour in Pinot Noir wines. This is a relatively common (if controversial) technique in the wine industry, but its exact impacts are still the subject of heated debate (Butler 1981; Dambergs et al. 2012; de la Barra et al. 2005; Goode 2012). It is hypothesised that the addition of stems will impact the phenolic profile, and hence the colour, of Pinot Noir wines.

(c) To test the hypothesis that commercial Pinot Noir wines originating from regions of south-eastern Australia could be identified by metabolomic testing to give a profile of *terroir* relating to both soil, climate, and geology; in particular mineral (metal ion) content, which may relate to the soil types of the regions, and key organic compounds, which may relate to the climate and vintage conditions, are thought to be important. The wines were then analysed via spectrometry, ICP-MS, and NMR. It was hypothesised that the wines could be classified by region based on their metabolomic profile. This is presented as Chapter 4, and has been submitted for publication as a paper.

Finally, Chapter 5 presents some overall conclusions, and summarises suggestions for future study.

Chapter 2. The impact of temperature on berry and wine colour in Pinot Noir in a cool climate (Lenswood, Adelaide Hills) vineyard

2.1 Introduction

This study was run over two years, with the first year functioning primarily as a pilot study. In both cases, the hypothesis was identical: that warmer temperatures would produce Pinot Noir grapes and wine that were lighter in colour.

The study used the heating system devised by Sadras and Soar (2009), who found that sugar and anthocyanin accumulation were decoupled at elevated temperatures. This previous study, however, looked at Barossa Shiraz grown on a flat site. This heating system had never before been used for Pinot Noir in a cool region (e.g., the Adelaide Hills), or on a sloped site.

Some data has been pooled from both years of the study, to overcome the lack of true replication, a problem discussed in the Methods section.

2.2 Aims

This study aimed to investigate the impact of temperature on colour in *Vitis vinifera* L. 'Pinot Noir' grapes and wine, using the passive heating system devised by Sadras and Soar (2009). It was hypothesised that increased temperatures would decrease the colour of grapes and wine.

2.3 Methods

2.3.1 Vineyard trial

A trial plot was set up on in a vineyard near Lenswood, South Australia (34°53′31.9″S 138°49′34.9″E) on a north facing slope at 536 m above sea level, planted with *Vitis vinifera* L. 'Pinot noir' clone D5V12.

Two treatments were set up as per Sadras and Soar (2009)— a normal area of vines (designated the 'control treatment'), and adjacent to this an area of vines that

were warmed passively (the 'heated treatment', see Figure 2.1). The latter treatment comprised clear polycarbonate panels on steel frames, designed to sit over the vines and increase the solar warming effect, hence with the aim to provide increased temperatures during the day, with little impact on minimum temperatures at night (Sadras & Morán 2012; Sadras & Soar 2009). By using this 'heated treatment' adjacent to the control, the impact of confounding factors such as soil, sunlight, or altitude could be minimised.

Three pseudo-replicates of each were set up across the slope, with a heated and control treatment each at the base, middle and top of the slope (see Figure 2.2, note that the ground at the top of the slope was relatively flat). Each pseudo-replicate contained six vines, and a gap of one row was left between heated and control treatments. However, due to potential microclimate differences between the top, middle, and base of the slope, these were determined not to be true replicates. Consequently, data from both years have been pooled for some of the statistical analyses, in order to provide more robust statistics based on an unreplicated trial over two seasons.

The study area received the same viticultural treatments (mowing, and spraying with wettable sulfur, dipel, and copper sulfate) as the rest of the vineyard. Temperature and relative humidity were logged every 15 minutes using TinyTag Plus 2 TGP 4500 (Gemini Data Loggers Ltd, Chichester, West Sussex, UK) dataloggers. The berry sampling strategy was as described in section 2.3.3, Analysis. Additionally, during the 2015 growing season photographs were taken of the flowers and berries in each replicate from flowering onwards.

Figure 2.1: Glasshouse panels set up in the Lenswood vineyard, Aug. 2013, showing top of slope.

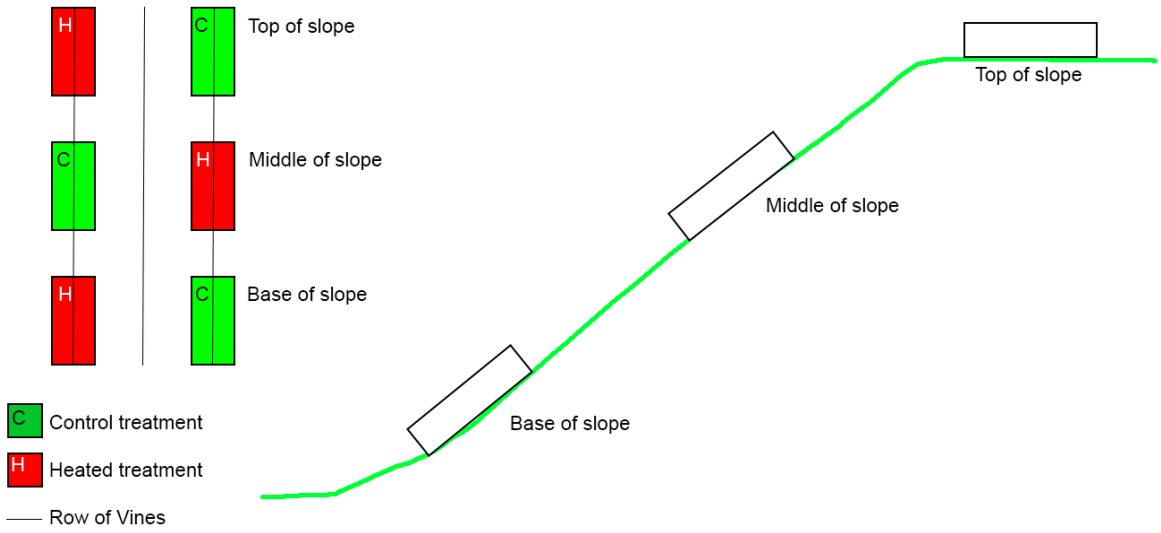


Figure 2.2: Diagram of vineyard experimental area, with treatments and their location on the slope indicated

2.3.2 Winemaking

The grapes were harvested between 24 and 25 Brix.

Winemaking in the first year was conducted in food grade plastic buckets. The grapes were manually destemmed, and rotten, under- or over-ripe berries were discarded. Healthy grapes were moved to 5 L plastic food grade buckets (Menzel Plastics, Adelaide, South Australia), and 50 ppm sulfur added as potassium metabisulfite. They were manually crushed using a ferment plunger, cold soaked for 20 hours in a room held at 0 °C, then inoculated with the yeast *Saccharomyces cerevisiae* EC1118 at a rate of 0.5 g L^{-1}. The ferments were plunged twice daily and kept in a room at 18 °C. They were pressed after thirteen days. They were then inoculated with malolactic culture following the manufacturer's instructions, and malolactic ferment was carried out in a room kept at 23 °C. Once malolactic fermentation was judged to be finished, a further 50 ppm sulfur was added and the wines were cold stabilised, then bottled in 375 mL green glass bottles sealed with screwcaps.

Winemaking in the second year was conducted as per Dambergs et al. (2012) and Liccioli (pers. comm., 2014). Grapes were manually destemmed, and mouldy, under- or over-ripe berries were discarded. The yield was split into three 1.3 kg fermentation replicates per vineyard replicate, and 50 ppm sulfur added as potassium metabisulfite. Each batch of berries was crushed manually in a plastic bag then placed into a 1.5 L Bodum French Press coffee plunger, and inoculated with the yeast *S. cerevisiae* EC1118 at a rate of 0.5 g L^{-1}. The ferments were plunged daily and kept in a room at 18 °C.

Each ferment was pressed when less than 1 g L^{-1} residual sugar remained, using the coffee pot plunger with a 4 kg weight to ensure even pressing, left in place for five minutes, then the liquid was decanted into an argon-filled glass bottle. The pressing was dug out and re-pressed, and the remaining liquid added to the bottle. Each bottle was topped up with argon gas, then sealed. The must was left 48 hours to settle, then decanted off the lees into a fresh glass bottle containing argon gas. A further 50 ppm of sulfur dioxide was added, and glass marbles added to bring the level of the wine into the neck of the bottle. Malolactic fermentation was not undertaken in 2015. The wines were cold stabilised, then bottled in 200 mL brown glass bottles.

2.3.3 Analysis

Analysis was undertaken of both grapes and wines.

Berry analysis: In 2014, berries were sampled initially every seven days from immediately before *véraison* to harvest (with a sample of 150 berries), then every ten days (with a sample of 100 berries), as the low yields that characterised the harvest became apparent. In 2015, berries were sampled every eight to nine days from immediately before *véraison* to harvest, with a sample of 150 berries per replicate. In both years, 50 berries were used immediately to determine total soluble solids (TSS), pH, and TA. TSS were determined using a refractometer, TA and pH were determined using a Crison Compact Titrator (Crison Instruments SA, Barcelona, Spain). The remainder were frozen at -20 °C for analysis using a Cintra 4040 UV/Vis spectrophotometer (GBC Scientific Equipment, Melbourne, Australia), using Iland's

technique for assay of anthocyanins and total phenolics of grape berries, with minor modifications (Iland et al. 2004, pp. 44-48; Iland et al. 1996).

Fifty berries were counted out while frozen, and then were left to thaw for approximately one hour. They were then weighed, and transferred to a 125 mL plastic beaker. The berries were homogenised using an Ultra-Turrax T25 homogeniser (IKA Works GmbH & Co, Staufen, Germany) for 90 seconds (the machine was run for 60 seconds, then the mixture stirred to check for remaining whole berries, and then homogenised again for a further 30 seconds; first year), or using an immersion blender for 30 seconds (second year). The homogenate was mixed thoroughly, then a scoop of approximately 1 g of homogenate, recorded as the 'weight of the homogenate taken for extraction', was placed in a 15 mL centrifuge tube. Three such replicates per sample were made. A 50/50 v/v mix of MilliQ water (acidified to pH 2.0 with concentrated hydrochloric acid) and analytical reagent grade ethanol was prepared, and 10 mL was added to each centrifuge tube. The mixture was then extracted for two hours, with the tubes shaken vigorously every five minutes. The centrifuge tubes were then centrifuged at 3500 rpm for 10 minutes. The supernatant (called 'the extract') was removed to a fresh centrifuge tube. Then, 1 mL of this extract was then added to 10 mL of 1.0 M HCl in another centrifuge tube and mixed thoroughly. The volume of the remaining extract was measured (the volume of the remaining extract plus the 1 mL that was acidified was defined as the 'total extract volume').

After three hours, the absorbance of the diluted HCl extract was read at 700, 520, and 280 nm (Iland et al. 2004; Iland et al. 1996) in a 1 cm quartz cuvette, using a Cintra 4040 UV/Visible spectrophotometer. Readings were also taken at 420 and

620 nm (Glories 1984; Ivanova, Vojnoski & Stefova 2012). Each sample was read three times.

An additional sample from the extract was used to analyse pigment colour and density by the CIELab method (Liang et al. 2011; Ohno 2000), using a Cintra 4040 UV/Visible spectrophotometer. A narrow (1 mm path length) quartz cuvette was used for this. CIELab parameters, originally devised for the colour printing industry, provides an analysis of colour and density expressed as a three-dimensional 'colourspace'. The three CIELab coordinates—designated as parameters L*, a*, and b*—were measured for all CIELab analyses (grape and wine). L* defines 'lightness' (i.e. colour density) and is the vertical axis, with 0 as absolute black and 100 as absolute white; a* and b* are perpendicular horizontal axes, with a* defining colour in terms of green (negative values) to red (positive values), and b* blue (negative values) to yellow (positive values) components (Cesa et al. 2017; Liang et al. 2011). Each sample was read twice.

Wine analysis: Wine samples were analysed via CIELab (Liang et al. 2011; Ohno 2000) using a Cintra 4040 UV/Visible spectrophotometer, using the CIELab colourspace as defined above (section 2.3.3). Each replicate ferment was sampled once, and each sample was read three times (technical replication).

Somers Analysis was also undertaken using a Cintra 4040 spectrophotometer, to determine total phenolics, total anthocyanins, and colour density of the experimental wines (Mercurio et al. 2007; Somers 1971; Somers & Evans 1974, 1977). The protocol was as per Mercurio et al. (2007), but only total phenolics, total anthocyanins, and colour density were measured. Each replicate ferment was sampled once, and each sample was read twice.

Statistical analyses: Statistical analyses were undertaken using Microsoft Excel, SAS StatView (SAS, Cary, NC, USA), and GNU R (R Core Team 2019). The GNU R package 'segmented' (Muggeo 2003, 2008) was used to examine the relationship between TSS and factors such as anthocyanin levels during ripening. Significance was determined via analysis of variance (ANOVA) using a two-factor cross-factored unreplicated model (Doncaster & Davey 2007), and the significant difference limit was defined as probability (p) < 0.05. Graphs showing means and standard errors were produced using a modified version of the 'PlotMeans.R' script devised by Doncaster (2017). Vapour pressure deficit (VPD) was calculated from temperature and relative humidity using the 'RHtoVPD' function in the GNU R package 'plantecophys' (Duursma 2015), an air pressure of 101.3 kPa was assumed given the elevation of 536 m above sea level.

2.4 Results and discussion

2.4.1 Use of passive heating system

Temperature sensors showed clear differences in maximum temperatures between the heated and control treatments at the top of the slope, but it was less clear cut for the middle and base of the slope (see Figure 2.3). Overall, the averages of the temperatures of the heated treatment were higher than those of the control treatments. Negative temperatures show where the heated treatment was cooler than the control treatment, because the heating system also shielded the vines from fluctuations and rapid changes in temperature.

Temperature differences were noticeable between locations on the slope (see Figure 2.3 a & c), with the overall seasonal average temperature differences observed

showed the heated treatment at the top being 1.6 °C warmer than the control treatment, the middle 0.2 °C warmer, and the base 0.4 °C warmer across the entirety of the study (September 2013-March 2015).

The differences in maximum temperatures (i.e., peak temperatures) observed over the same period (see Figure 2.3 b & d) showed the heated treatment at the top of the slope peaking at 9.8 °C warmer than the control treatment, the middle 18.9 °C warmer, and the base 13.3 °C warmer.

VPD was also affected, with the heated treatment at the top of the slope having a VPD of -0.127 kPa compared with the control treatment, the middle a VPD of -0.154 kPa, and the base a VPD of 0.0122 kPa.

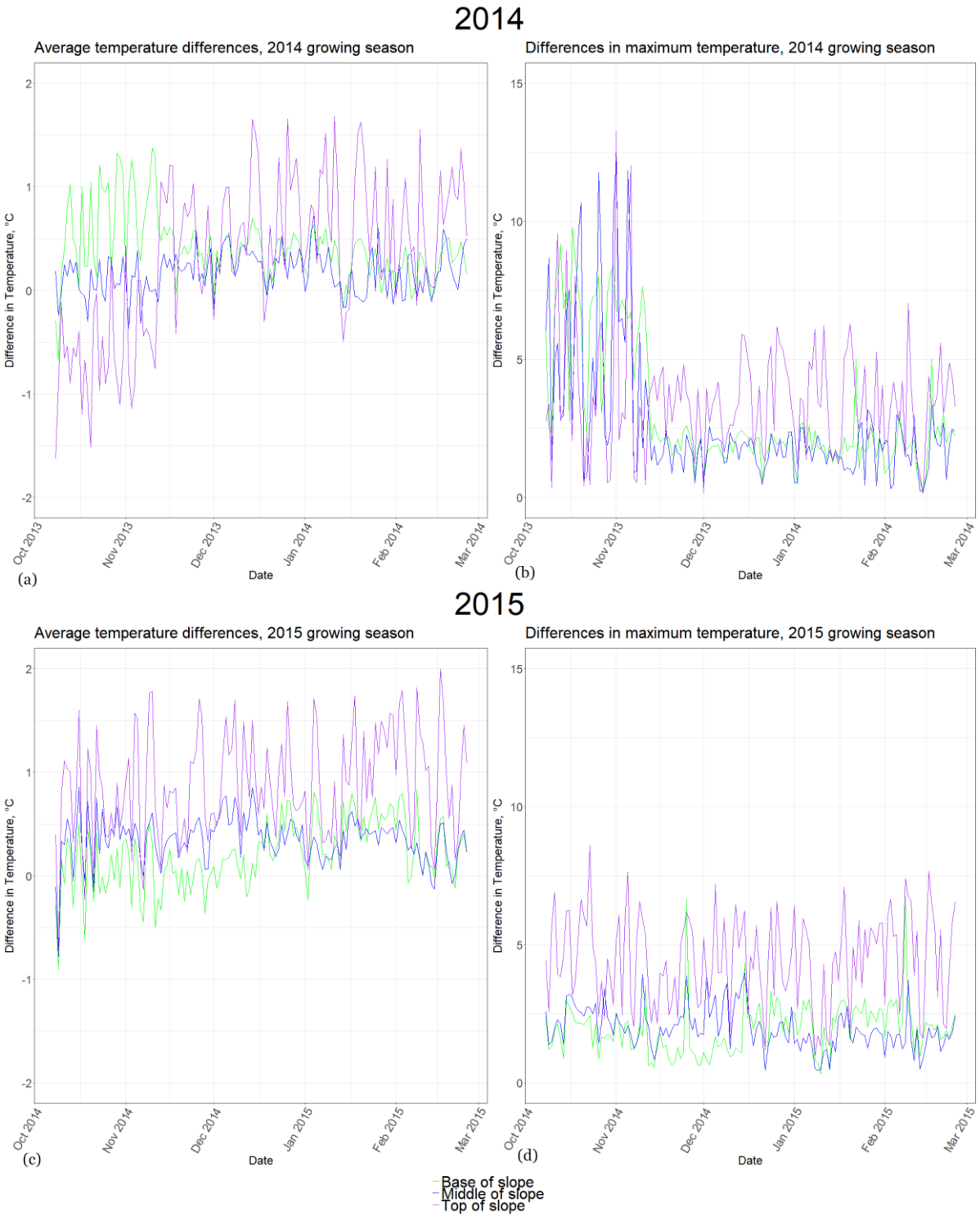


Figure 2.3: Average temperature differences (a & c) and differences in maximum temperature (b & d) between heated and control treatments.

X-axis shows dates of average readings, (2014: 8 Oct 2013 to 25 Feb 2014, 2015: 8 Oct 2014 to 25 Feb 2015, i.e. Southern hemisphere growing season, a & b; c & d)

2.4.2 Berry analysis

Data from two years were pooled (see section 2.2.1), and the relationships between various grape ripeness traits (see Figure 2.4 and Figure 2.5) and TSS were analysed. Figure 2.4 shows the relationships between TSS and anthocyanins of the grape extracts. As described by Sadras and Morán (2012), an initial lag phase can be detected during which anthocyanin levels of berries do not noticeably increase as TSS accumulate, followed by an approximately linear phase during which TSS and anthocyanins increased roughly in parallel (Figure 2.4a). A piecewise regression was fitted to the data, which placed the break point between the lag phase and the linear phase at 10.6 ±0.7 °Brix. Following the model of Sadras and Morán (2012), the data was restricted to the point after the break point, a linear regression fitted (Figure 2.4b), and the residuals for both the control and heated treatment were calculated (Figure 2.4b inset).

In agreement with the findings of Sadras and Morán (2012), the heated treatment had a lower concentration of anthocyanins at the same level of TSS than the control treatment, as shown by the negative mean residual for the heated treatment and the positive mean residual for the control treatment. This suggested that the warmer temperatures had decoupled the accumulation of sugar and anthocyanin ($p < 2.2 \times 10^{-16}$, see Appendix 1.1). The relatively large standard errors (represented as error bars on the inset graph on Figure 2.4b) demonstrate the high variability inherent in a large field experiment such as this. The geometry of the slope also introduced a source of variability in temperature control, as noted above, presumably from allowing heated air to rise under the panels up the slope (the

heating panels were originally designed for a flat site). This is discussed further below.

Figure 2.5 and Figure 2.6 show the relationship between various other grape ripeness traits and TSS. In Figure 2.5, the heated and control treatments have been separated, so that differences between the two can be seen, as they appear to form different sets. Bilinear models roughly analogous to that seen in Figure 2.4 are seen in Figure 2.5, and in Figure 2.6 a, but not in Figure 2.6 b or c. However, the break point varies for each parameter, and the validity of the linear models is doubtful.

These differences were also evident visibly, and are recorded in the photographs of flowers and berries in each replicate taken from flowering onwards (Figure 2.7: photographs of flowers and berries in each replicate taken from flowering onwards, documenting changes in phenology). Differences between the heated and control treatments are clearly visible, with *véraison* occurring earlier in the heated treatments than the control treatments. The heated treatments were quicker to ripen, and reached the target sugar levels earlier than the control treatments.

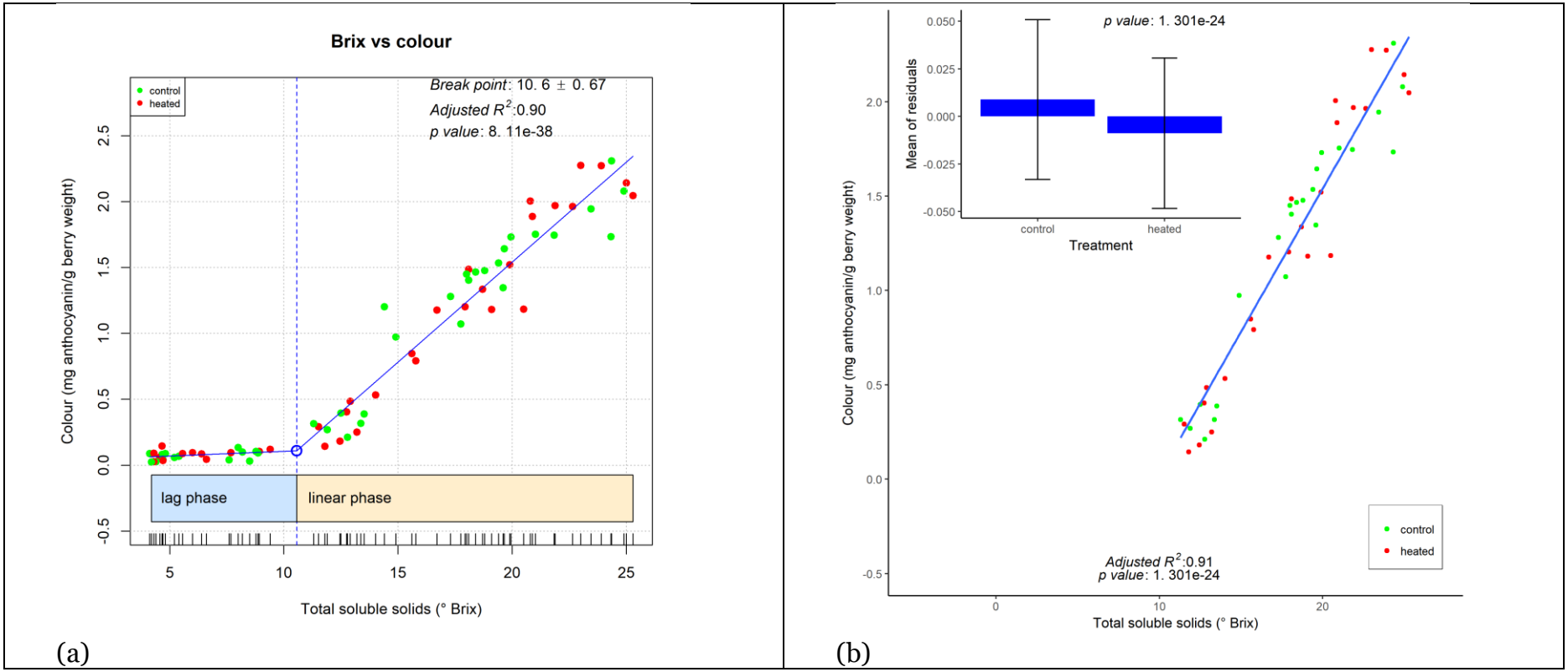


Figure 2.4: (a) Scattergraph of total soluble solids vs colour.

The blue solid line is the piecewise regression, and the blue dotted line shows the break point at 10.6 ±0.7 °Brix. (b) Linear phase relationship between TSS and anthocyanins, the blue line represents the fitted linear regression. The inset shows the differences in regression residuals for heated and control treatments, error bars represent two standard errors.

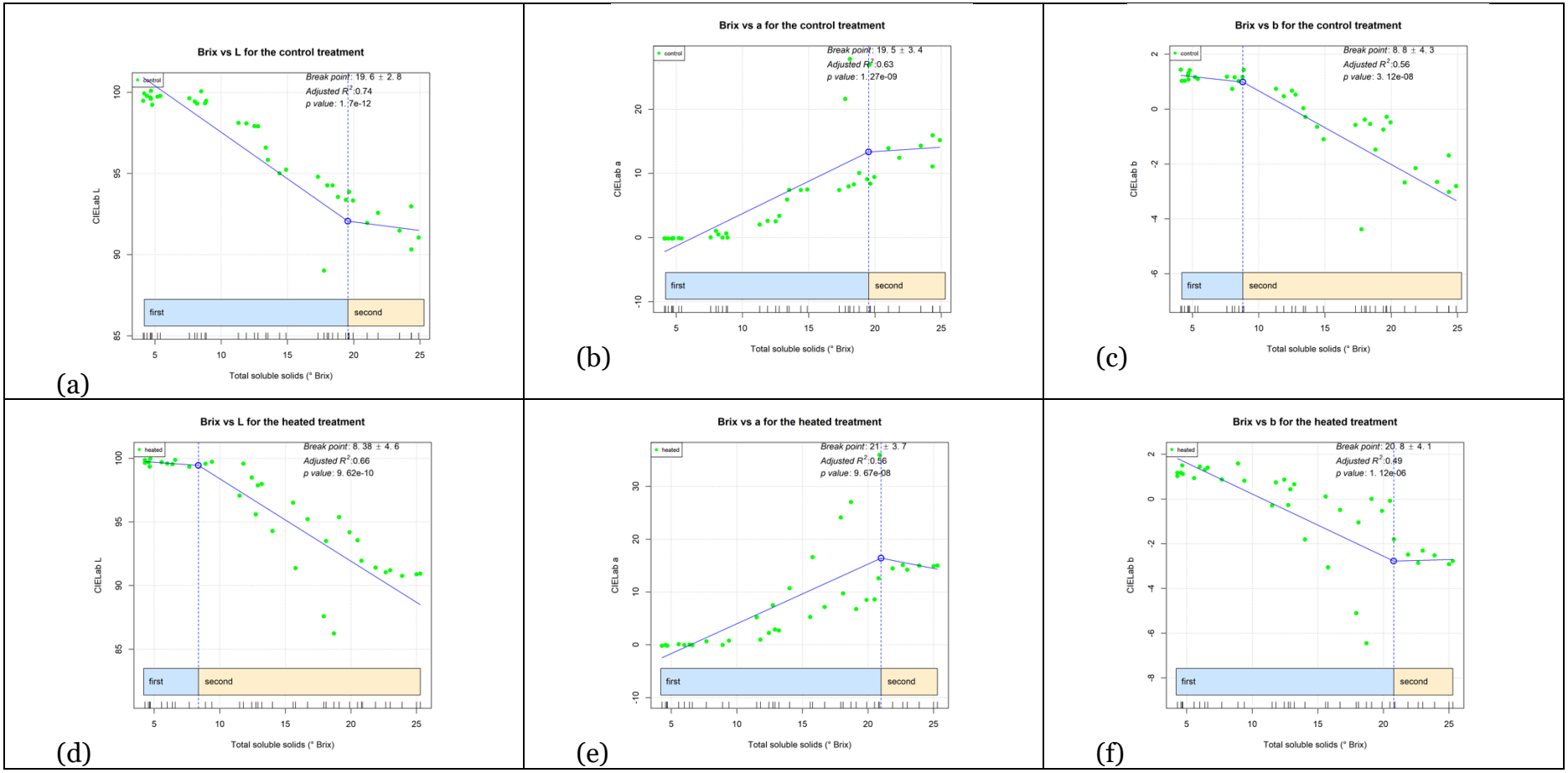


Figure 2.5: Scattergraph with linear model for total soluble solids vs CIELab parameters (a) L*, (b) a*, (c) b*, for the control treatment, and CIELab parameters (d) L*, (e) a*, (f) b*, for the heated treatment.

The blue solid line is the piecewise regression, and the blue dotted line the break point.

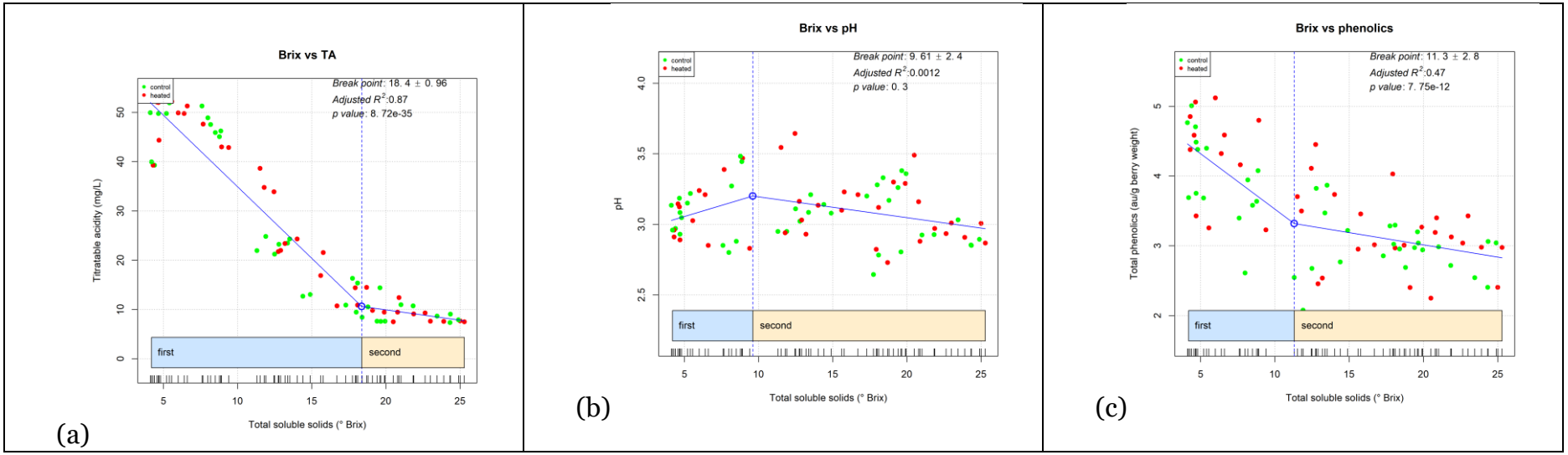
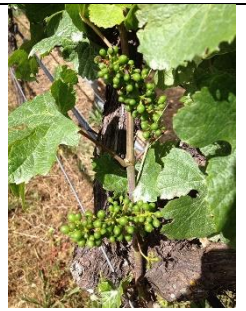


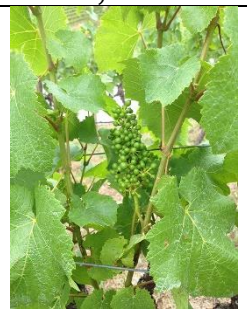
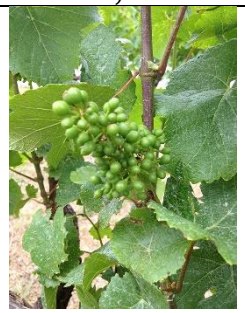
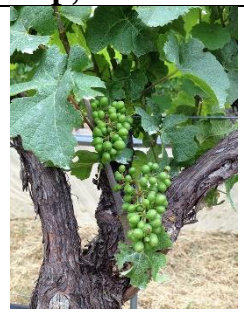
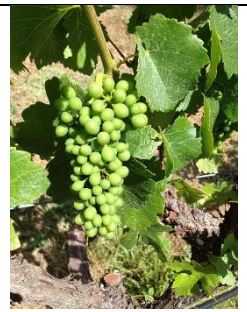
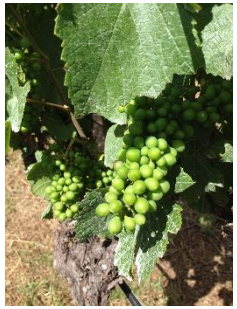
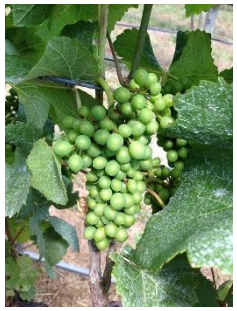
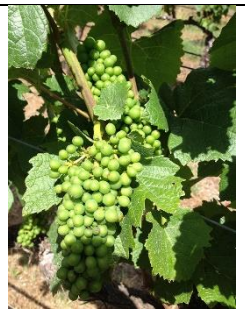
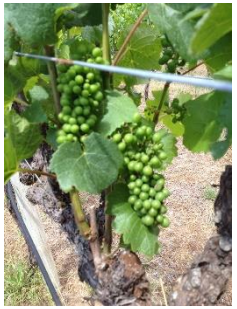
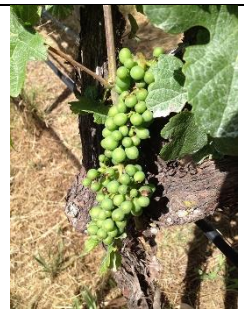
Figure 2.6: Scattergraph with linear model for total soluble solids vs (a) Titratable acidity, (b) pH, and (c) total phenolics.

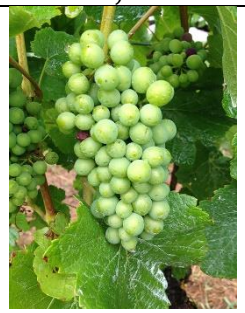
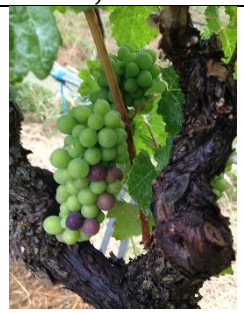
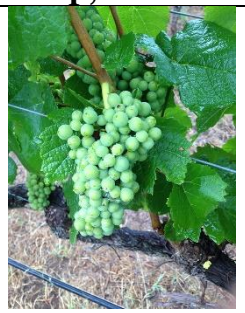
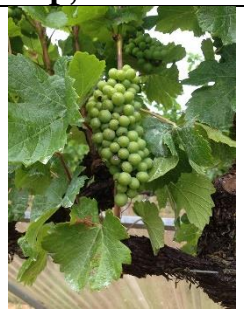
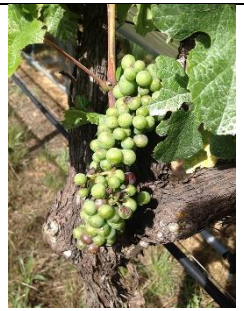
The blue solid line is the piecewise regression, and the blue dotted line the break point.

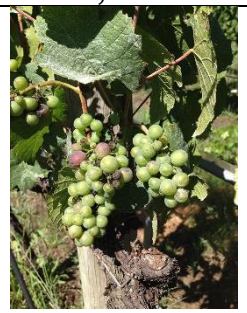
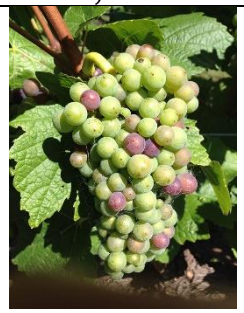
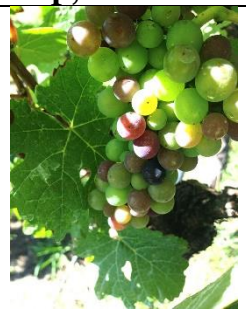
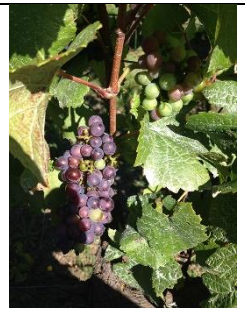
Figure 2.7: photographs of flowers and berries in each replicate taken from flowering onwards, documenting changes in phenology

Figure is on next page.

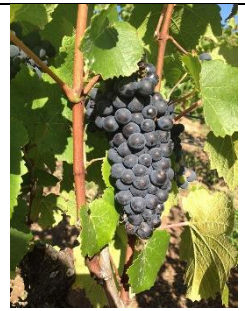
Date	Base, control	Base, treatment	Middle, control	Middle, treatment	Top, control	Top, treatment
11/11/2014	 	 	 	 	 	 
26/11/2014	 	 	 	 	 	 

Date	Base, control	Base, treatment	Middle, control	Middle, treatment	Top, control	Top, treatment
5/12/2014	 	 	 	 	 	 
16/12/2014	 	 	 	 	 	 

Date	Base, control	Base, treatment	Middle, control	Middle, treatment	Top, control	Top, treatment
8/1/2015	 	 	 	 	 	 
15/1/2015		 	 	 	 	 

Date	Base, control	Base, treatment	Middle, control	Middle, treatment	Top, control	Top, treatment
22/1/2015	 	 	 	 	 	 
30/1/2015	 	 	 	 	 	 

Date	Base, control	Base, treatment	Middle, control	Middle, treatment	Top, control	Top, treatment
9/2/2015						
						
17/2/2015						
						

Date	Base, control	Base, treatment	Middle, control	Middle, treatment	Top, control	Top, treatment
25/2/2015	 	 	 	Harvested 24/02/2015	Harvested 20/02/2015	Harvested 17/02/2015
At harvest (variable dates, as indicated)	  27/02/2015	  26/02/2015	  25/02/2015	  24/02/2015	  20/02/2015	  17/02/2015

2.4.3 Wine analysis

After the grapes were harvested, wines were produced as detailed in the Methods section. Ferments proceeded smoothly, with sugars declining rapidly and temperatures remaining steady. While wine was produced in both 2014 and 2015, the results from 2014 were inconclusive in part due to a poor season for the region with uneven ripening (Craig Markby 2014, pers. comm.), and hence are not included in this analysis.

The lightness of the wine colour (CIELab parameter L*) showed significant differences ($p < 0.05$) between different locations on the slope (i.e., top, middle, or base of slope), with the middle of the slope producing wines that were somewhat lighter than either the top or base of the slope; but no significant differences were observed between the heated and control treatments (see Appendix 1.2). This suggests that wine lightness (L*, see Figure 2.8), or colour density, may not be impacted by temperature, at least not in terms of the changes in temperature simulated by this present study.

CIELab parameter a* showed significant differences ($p < 0.05$) between the heated and control treatments, with the heated wines being slightly more positive (redder) than the control wines; but no significant differences were observed between different locations on the slope (see Appendix 1.2).

CIELab parameter b* also showed significant differences ($p < 0.05$) between the heated and control treatments, as well as differences based on location on the slope (see Appendix 1.2). This was also noticeable in the graphs (Figure 2.8) produced from the same data.

It is of interest that parameter b* (blue-to-yellow (Cesa et al. 2017; Liang et al. 2011), see Figure 2.8) seems most impacted by warmer temperatures, with parameter a* (red-to-green (Cesa et al. 2017; Liang et al. 2011), see Figure 2.8) also being impacted but to a lesser extent. Parameter L* (lightness), in contrast, seemed to be most impacted by the location on the slope, perhaps due to differences in UV levels, hours of sunlight, or soil.

As with the CIELab data, significant differences ($p < 0.05$) were seen between the heated and control treatments for the Somer's analysis data (see Appendix 1.3). Again, significant differences ($p < 0.05$) were also observed based on the location of the treatment on the slope (i.e., top, middle, or base of slope). This was true for total anthocyanins, colour density, and total phenolics. This is also noticeable in the graphs (Figure 2.9) produced from the same data.

The heated treatment showed higher levels of total anthocyanins and total phenolics than the control. Interestingly, significant differences ($p < 0.05$) were also observed based on the location of the treatment on the slope, with the base of the slope showing the highest levels of anthocyanins and total phenolics, followed by the top, and with the middle showing the lowest levels. Colour density was higher in both the top and base of the slope than the middle. While differences in temperature between the top, middle, and base of the slope were subtle, they were noticeable. This was in contrast to the results of the Bodum press fermentation trial (Chapter 3), and suggests that even such small differences in level on the hillside may be important—i.e. that these effects are highly site-specific. These differences are not obvious in the graphs (Figure 2.9), however.

These differences are only noticeable if the relevant heated and control treatments are separated out by their location on the slope. As with the CIELab data,

and again in contrast to the results of the Bodum press fermentation trial (Chapter 3), this indicates that site (especially the related, minor differences in temperature) may be highly important in determining wine colour and phenolic contents.

In particular, the wines from the heated treatment were slightly redder (higher mean a* values, p 2.741×10^{-4}) and slightly bluer (lower mean b* values, p 2.231×10^{-9}) than wines from the control treatment, i.e. heated treatment wines were slightly more coloured, though lighter in colour density. They also had slightly higher anthocyanin levels (p 0.02616, though the range overlaps considerably with that of the control treatment) and slightly higher total phenolic levels (p 2.72×10^{-13}) than wines from the control treatment.

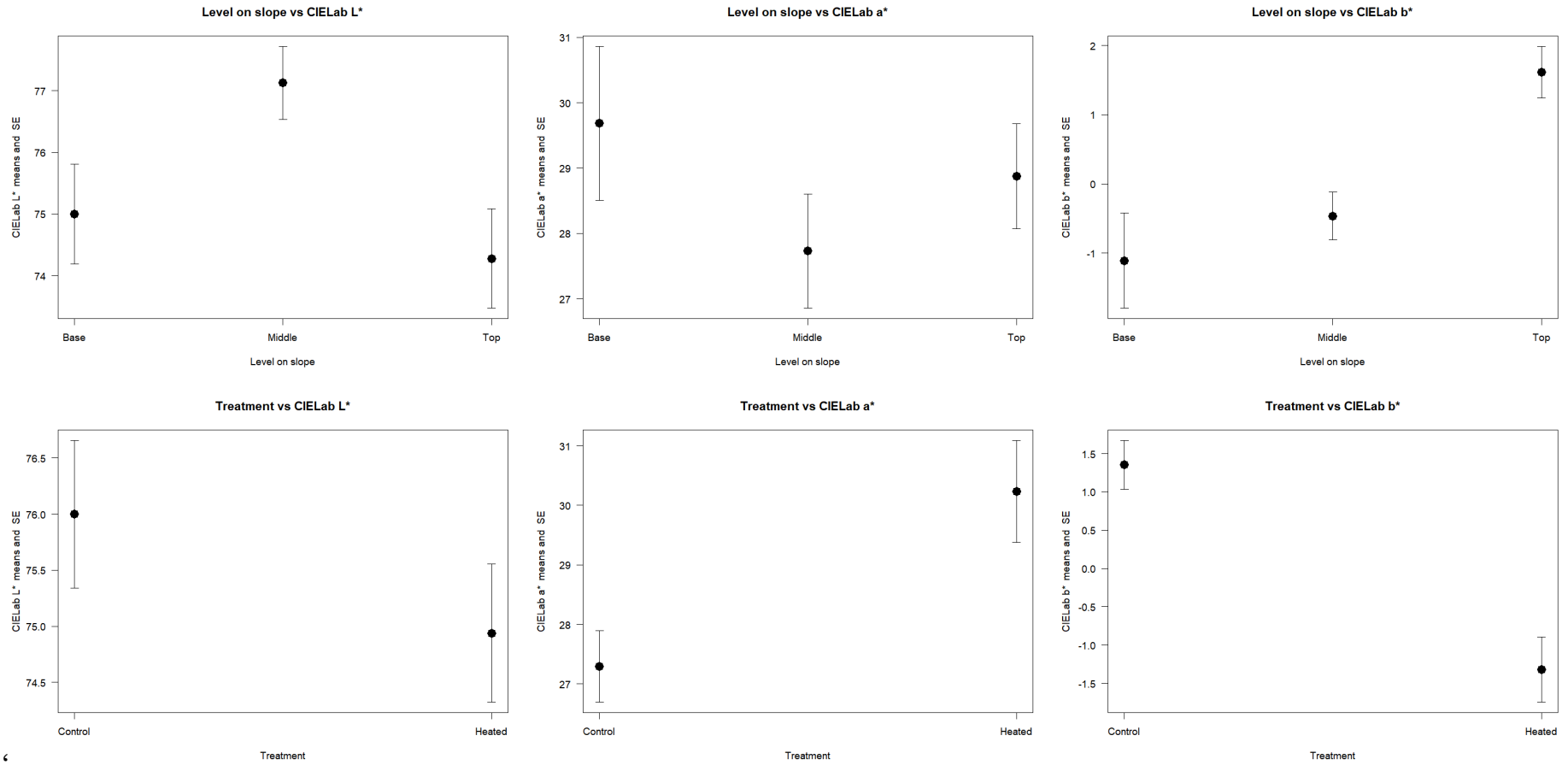


Figure 2.8: Heated treatments versus control treatments showed significant differences between the wines produced from the heated and control treatments for a* and b*, and between levels on the slope for b*. Each replicate ferment was sampled once, and each sample read twice.

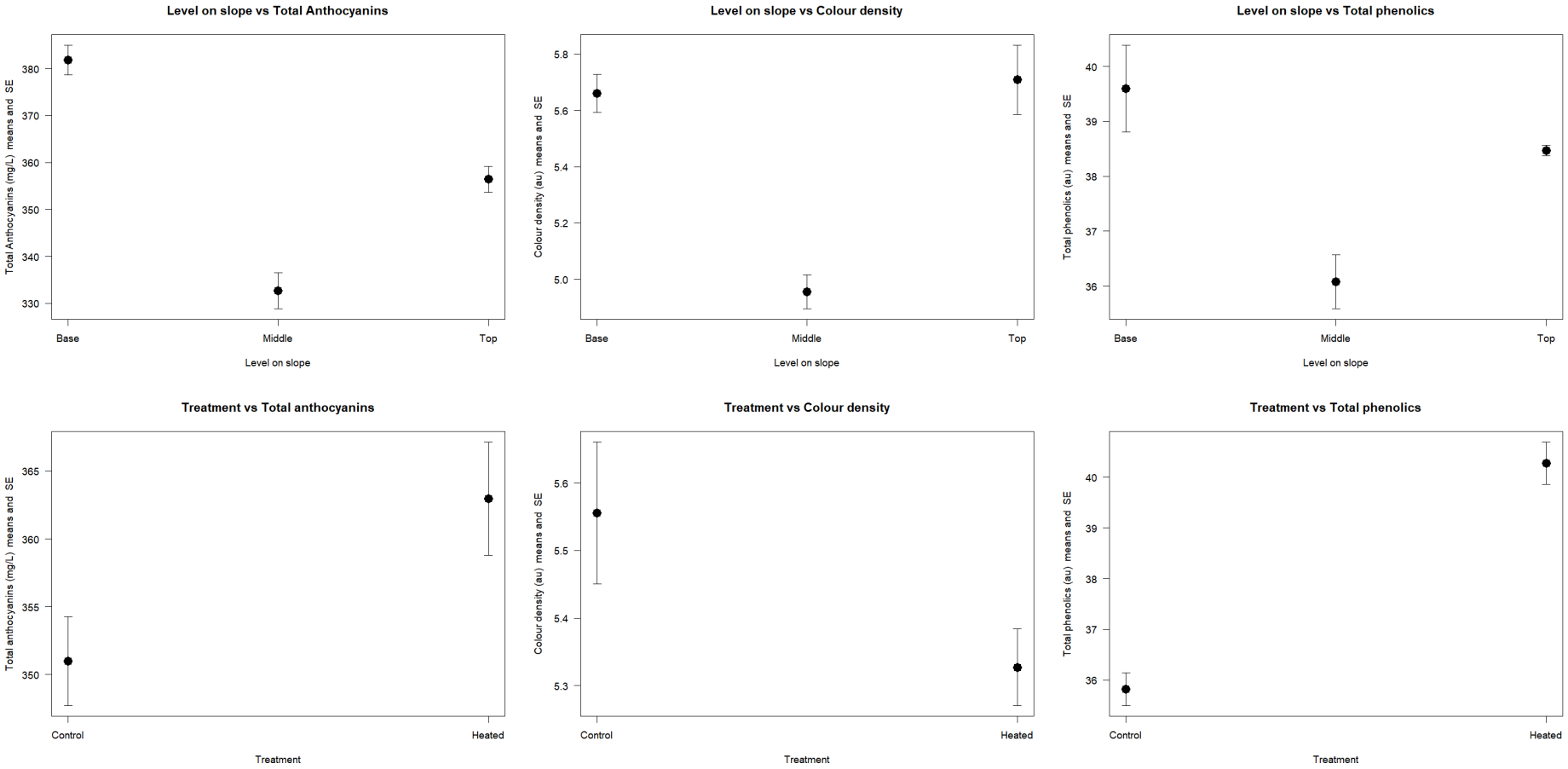


Figure 2.9: Heated treatments versus control treatments showed significant differences between the wines produced from the heated and control treatments, and between levels on the slope, for total anthocyanins, colour density, and total phenolics. Each replicate ferment was sampled three times, and each sample was read twice.

2.4.4 Further discussion

The questions raised by these results are:

- What impact will the factors of colour and phenolic composition have on wine quality?
- How important is temperature in determining wine colour and density?

The importance of phenolic compounds—both in terms of colour compounds, and of tannins—has long been noted (Dambergs et al. 2012). Colour has long been an important factor in determining consumer acceptance of a wine (Dambergs et al. 2012; Parpinello et al. 2009). Previous wisdom suggests that cooler climatic conditions would favour colour development (Kliewer 1970; Kliewer & Torres 1972; Mori et al. 2007; Sadras & Morán 2012; Yamane et al. 2006). This project noted the same decoupling of anthocyanins and sugars as seen in Sadras and Morán (2012), with higher anthocyanin concentrations at the same sugar level in the cooler control treatment than the heated treatment. A similar effect was noted in Bonada et al. (2015), where flavonoids and colour were reduced in berries grown under high temperatures.

The results were less clear for wines than for berries. As noted in Bonada et al. (2015) and Bindon, Dry and Loveys (2008), berry composition differences are only partly reflected by differences in wine composition. As discussed in Section 2.3.3, wines produced from the heated treatment seemed slightly redder (positive a* values) and bluer (negative b* values), i.e. more purple, with increased levels of total phenolics (and perhaps of total anthocyanins), than those produced from the control treatment (see Figure 2.8 and Figure 2.9). This may in part be due to the higher levels of total

phenolics observed in the heated treatment. This is somewhat similar to the findings of Bonada et al. (2015), where wines produced from grapes from their treatments (artificially heated and water deficit) were redder (higher a*) than those of their control treatments; in contrast, they found the wines from the treatments to be darker (lower L*) and more yellow (higher b*).

Boulton (2001) noted that early studies on copigmentation found tannin additions shifted the colour of malvidin 3-monoglucoside towards blue, rather than its usual red colouration, which would appear to be the case in this study as well. Boulton attributed this phenomenon to monomeric cofactors and dimers, rather than tannins *per se*; however, these would be included in any measure of total phenolics, and hence the higher levels of total phenolics in the heated treatment may explain the more negative values for the CIELab b* parameter. The reason for the increase in total phenolics is less obvious, however, with most previous research suggesting that total phenolics may be more influenced by vine light interception than by vineyard temperature (Nicholas et al. 2011; Song et al. 2015). In contrast, Nicholas et al. (2011) reported that vineyard sites experiencing cooler temperatures during the previous autumn produced wine with significantly *higher* total phenolics, the opposite of the results observed here.

The importance of site is supported by this study, in particular the differences between the top and base of the slope, where CIELab colour, total anthocyanins, colour density, and total phenolics vary between the top, middle, and base of the slope—though, in most cases, without clear, obvious patterns. Differences in ripening time based upon location on the slope had already been recorded by the vineyard management (Craig Markby, 2013 pers. comm.), and were also noted during the course of this study. This echoes experience in other wine regions, where differences

in vineyard site, and within a particular vineyard, have long been considered to impact wine quality and composition (de Andrade et al. 2013; Nicholas et al. 2011; Roullier-Gall, Boutegrabet, et al. 2014; Roullier-Gall, Lucio, et al. 2014; van Leeuwen et al. 2004; van Leeuwen & Seguin 2006; Young-Shick 2011).

A final point would be to question how these changes in colour and phenolics are likely to be perceived by consumers. It has been presumed that any factor which increases colour density may make the wine more acceptable to consumers (Dambergs et al. 2012; Parpinello et al. 2009). However, more recently, wine critic Jamie Goode has noted an increase in interest in lighter reds among wine consumers, including lighter coloured Pinot Noirs (Goode 2015).

2.4.5 Suggestions for future work

A future study conducted on a flatter vineyard site could provide better replication of heated and control treatments. This may provide more robust data, and perhaps better separate the heated and control treatments, allowing a clearer narrative to be drawn. Such sites are difficult to locate in the Adelaide Hills, hence replicating this study in another cool climate region (e.g., the Mornington Peninsula or Tasmania) may be of interest.

There may be an optimal temperature for the accumulation of anthocyanins and cofactors, depending upon the variety of the wine grape. A study that examined anthocyanin accumulation at three or more different temperatures (cold, warm, and hot) may help to determine whether this is correct, and if so what the optimal for a

given grape cultivar would be. This may be one study where use of potted vines grown in glasshouses may be of potential use.

Finally, this study focussed exclusively on colour and phenolics. However, volatile compounds that produce wine flavours and aromas are also critical to determining wine quality, and hence consumer acceptance (Charters, Robinson & Harding 2015; Coombe et al. 2015). Future studies could investigate the impact of temperature on these critical wine quality factors, perhaps via use of sensory analysis and of gas chromatography (e.g. gas chromatography-mass spectrometry, and gas chromatography/olfactometry) (Francis & Newton 2005). Bonada et al. (2015) used similar methods to investigated Barossa Shiraz, and made use of sensory analysis to gain additional information on the differences between heated/water deficit treatment wines and control wines. The same methods could be used for a future study on Pinot Noir.

2.5 Conclusions

Differences between the heated and control treatments were clearly noticeable. The position on the slope appeared to contribute to the effect, suggesting that the impact of warming was highly site-specific.

As in Sadras and Morán (2012), elevated temperatures may have decoupled the accumulation of sugars and anthocyanins, with the heated treatment grapes producing lower anthocyanin levels than the control treatment grapes, for the same level of TSS. The results were less immediately obvious for the wines than for the grapes, with the wines from the heated treatment vines having higher total phenolics, and perhaps consequently lower (bluer) CIELab b* values.

This study suggests that climate change will likely impact the production of Pinot Noir in Australia. The impact may be difficult to predict, and it is possible that the increase in total phenolics—and hence blueness—may counteract other changes in colour. Further work to determine more precisely any likely impacts would be of interest.

Chapter 3. Fermentation trials

3.1 Introduction and Aims

Two small scale winemaking trials were undertaken using *Vitis vinifera* L. 'Pinot Noir' clone D5V12 grapes from a separate section of the same vineyard in Lenswood, South Australia, Australia as used for the study described in Chapter 2.

3.1.1 Aims: Bodum press fermentation trial

The use of French press coffee pots or 'Bodum' presses as fermenting vessels has been trialled successfully in other institutions (Dambergs et al. 2012), and was carried out for this study under the advice of Dr. Liccioli (pers. comm., 2014).

There were two aims:
(a) to test using commercial Bodum presses with plungers as a means of conducting small scale fermentations under controlled and reproducible conditions;
(b) to assess if location on the vineyard slope (which was a potential confounding factor for the heating treatment trials, Chapter 2) had any impact on wine colour in isolation from the desired effects of the heating system being trialled in that study.

It is hypothesised that the Bodum presses will be effective as fermentation vessels, and that the location on the slope will not affect wine character.

The experiment did not aim to determine whether Bodum press ferments would accurately represent larger scale ferments. The intention was purely to determine their usefulness for replicated, small-scale, experimental ferments.

3.1.2 Aims: Whole bunch ferments compared with destemmed ferments

This was undertaken to determine whether the inclusion of stems in fermentations would impact colour in Pinot Noir wines. This is a relatively common (if

controversial) technique in the wine industry, but its exact impacts are still the subject of heated debate (Butler 1981; Dambergs et al. 2012; de la Barra et al. 2005; Goode 2012).

It is hypothesised that the addition of stems will impact the phenolic profile, and hence the colour, of Pinot Noir wines.

3.2 Methods

3.2.1 Bodum press fermentation trial

Vitis vinifera L. "Pinot Noir" clone D5V12 grapes were harvested from an east facing slope at 516 m above sea level in a vineyard near Lenswood, South Australia, Australia (34°53′33.4″S 138°50′17.4″E), with three 'locations' based on the position on the slope (top, middle, and base of slope). Unlike for Chapter 2, where grapes were harvested at a specific target Baumé, the grapes were all harvested on the same date, and at varying sugar concentrations. Within each location, grapes were randomly split into three lots of 1.3 kg (i.e. nine in total). No sorting for under or over-ripe berries occurred.

These were then transferred to commercially available 35cm × 27cm resealable 'ziplock' plastic bags, and 50 mg L^{-1} sulfur dioxide added as potassium metabisulfite. The berries were then crushed by hand for three minutes, until the berries were all visibly crushed, in the plastic bags before being transferred to Bodum presses (Bodum Chambord Coffee Maker Stainless 1500 mL, Hospitality Products, Yatala, QLD, Australia). Each sample was then inoculated with the yeast *Saccharomyces cerevisiae* EC1118 (Lallemand, Rexdale, Ontario, Canada; yeast prepared from a live culture prepared by Dr Paul Grbin's laboratory) at a rate of 0.5 g L^{-1}, and 100 mg L^{-1} diammonium phosphate (DAP) for nitrogren supplementation.

Each ferment was kept in a temperature controlled room at 18 °C and was plunged five times twice daily. Sugar levels were monitored using a refractometer, and a commercially available enzymatic sugar assay (Vinessentials, Dromana, Vic, Australia) was used below 3 °Bx to confirm the fermentations had completed successfully at 0 °Brix. The ferments were then pressed, by placing a 4 kg weight on the top of the coffee pot plunger for five minutes. Then 40 mg L^{-1} sulfur dioxide was added as potassium metabisulfite and each wine bottled in a 375 mL green glass wine bottle, sealed by screwcap. These were stored at a constant 12 °C temperature.

Analyses of the colour of the small scale wines were recorded in terms of CIELab coordinates (Liang et al. 2011; Ohno 2000), using a Cintra 4040 UV/Visible spectrophotometer (GBC Scientific Equipment, Melbourne, Vic, Australia); CIELab colourspace coordinates are described in Section 2.3.3 (see Chapter 2). One sample was taken from each fermentation replicate, which was read three times (technical replication). The anthocyanin pigment malvidin-3-glucoside, responsible in part for the 'redness' of wine, was assayed by reversed phase HPLC, according to the methodology described by Peng et al. (2002). A Hewlett Packard Series 1100 HPLC (Agilent Technologies, Santa Clara, CA, USA) with a diode array detector (DAD) was used, with separation of analytes being achieved by elution through a Phenomenex Synergi 4 µm Hydro-RP 80 Å 150 x 2 mm Reverse phase column (Phenomenex Inc, Torrance, CA, USA). Three samples were taken from each treatment replicate, and with two replicates per sample. The samples were centrifuged at 10,000 rpm for 10 minutes before analysis. Statistical analysis (ANOVA) was undertaken using GNU R (R Core Team 2019).

3.2.2 Whole bunch ferments compared with destemmed ferments

Vitis vinifera L. "Pinot Noir" clone D5V12 grapes were harvested from an east facing slope at 516 m above sea level in a vineyard near Lenswood, South Australia, Australia (34°53′33.4″S 138°50′17.4″E). The grapes were randomly split into six lots of 2.5 kg. Three of these were destemmed (the destemmed control), and three were left as whole bunches (the stem ferment treatment). No sorting for under or over-ripe berries occurred.

Each treatment lot was then transferred to a commercially available (35cm × 27cm) resealable 'ziplock' plastic bag, and 50 mg L^{-1} sulfur dioxide added as potassium metabisulfite. The berries were then crushed by hand for three minutes, until the berries were all visibly crushed, in the plastic bag before being transferred to a 4 L food grade plastic bucket with a lid to exclude dust and insects (Menzel Plastics, Adelaide, South Australia, Australia) for open fermentation. The fermentations were inoculated with the yeast *S. cerevisiae* EC1118 (Lallemand, Rexdale, Ontario, Canada). This was prepared from active dried yeast at a rate of 0.2 g L^{-1} in a mixture of distilled water and grape juice, and 100 mg L^{-1} DAP for nitrogren supplementation. The ferment was plunged five times twice daily for the first seven days and kept in a constant temperature room at 23 °C, and then moved to an 18 °C constant temperature room until the eighth day and plunged five times once daily thereafter. Sugar levels were monitored using a refractometer, and a commercially available sugar assay (Vinessentials, Dromana, Vic, Australia) was used below 3 °Bx to confirm the fermentations were had completed successfully at 0 °Bx. Subsequently, each lot was pressed after ten days into 2 L demijohns using a 20 L bladder press (Hydro 20, Zambelli, Camisano Vicentino, Veneto, Italy). All

fermentations were then inoculated with *Oenococcus oeni* malolactic culture (VP 41, Lallemand, Rexdale, Ontario, Canada) following the manufacturer's instructions. The malolactic fermentation was carried out in a 23 °C constant temperature room. The malolactic fermentation was monitored using a commercial malic acid enzymatic analysis (Vinessentials, Dromana, Vic, Australia).

At this stage, 40 mg L^{-1} sulfur dioxide was added as potassium metabisulfite to each completed ferment, the wine acidified adding tartaric acid in powder form to pH 3.35 (since it is advisable to acidify red wines to a pH of 3.4 or below (AWRI n.d.)), then the wine bottled in 375 mL green glass bottles sealed with screwcaps. The wines were stored for nine months at a constant 12 °C temperature prior to analysis.

The wine samples were analysed via CIELab (Liang et al. 2011; Ohno 2000) and by the Somers' Methods (Mercurio et al. 2007; Somers 1971; Somers & Evans 1974, 1977), using a Cintra 4040 UV/Visible spectrophotometer. The protocol was as described by Mercurio et al. (2007), but only total phenolics, total anthocyanins, and colour density were measured. For CIELab, one sample was taken from each treatment replicant, and each sample was read three times (technical replication). For Somers Analysis, three samples were taken from each treatment replicant, and each sample was read twice. Statistical analysis (ANOVA) was undertaken using GNU R (R Core Team 2019).

3.3 Results and discussion

3.3.1 Bodum press fermentation trial

The fermentations using the Bodum press method went smoothly and quickly, with no obvious problems such as sluggish or stuck fermentations observed. Pressing occurred on the fifth day. There were no discernible levels of hydrogen sulfide

produced. No differences in fermentation dynamics between the ferments were noted.

As discussed in the methods section (section 3.2.1), the grapes for this trial were all harvested on the same day, and there were differences in ripeness between grapes from the top, middle, and base of the slope. However, this was not regarded as being a confounding factor in the trial.

The results of the CIELab analyses of ferment colour are shown in Figure 3.1. Parameters L* and a* showed no trends in 'lightness' or 'redness/greenness' respectively, with no significant differences between means predicted, as judged by the highly overlapping ranges. The coefficient of variation for L* suggested little variability between the replicates (1.17 % for wines from the base of the slope, 1.59 % for wines from the middle of the slope). In contrast, the wines from the top of the slope had a coefficient of variation of 8.23 %; however, this overlapped with the ranges of the wines from the base and middle of the slope, which suggested this would not be statistically significant (Figure 3.1). Parameter b* (blueness/yellowness) showed a trend towards increasing means going up the slope, although overlapping ranges suggested this would not be statistically significant. A statistical analysis confirmed that the *p* values were above 0.05 (above 5 %), and therefore that the CIELab colour differences between the top, middle, and base of the slope were not significant (Appendix 2.1).

No significant differences were notable in CIELab wine colour due to the position of the vines on the slope. This, by itself, would seem to suggest that the position of the vines on the slope does not have an impact on CIELab colour. It would also suggest that the position of the vines on the slope may not be a confounding factor for a study such as this (see Chapter 2).

Figure 4.3 shows the results for HPLC analysis of malvidin-3-glucoside concentrations in the ferments from the three slope positions. As with the values for 'blueness/yellowness' above (CIELab parameter b*), there was a trend discernible, with wine from the top of the slope having slightly higher malvidin concentrations. However, while the CIELab trends were not significant, a significant difference (F value of 6.000, *p* value of 0.01217) in pigment concentrations based on location on the slope was found (Appendix 2.2).

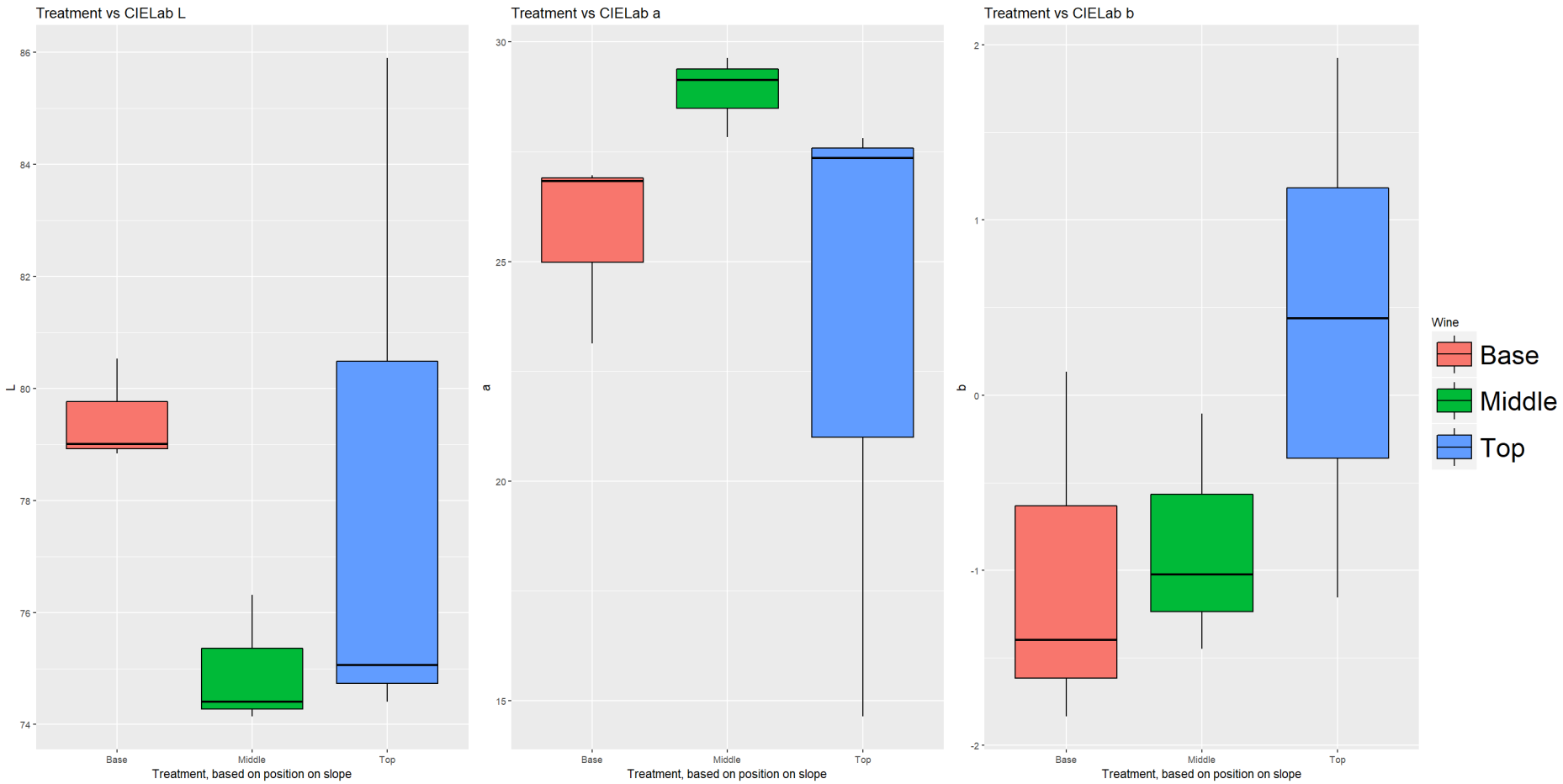


Figure 3.1: Wine CIELab results, shown as parametric box plots with mean, range (vertical line), and standard deviation (box). Each replicate was sampled once, and the same read three times.

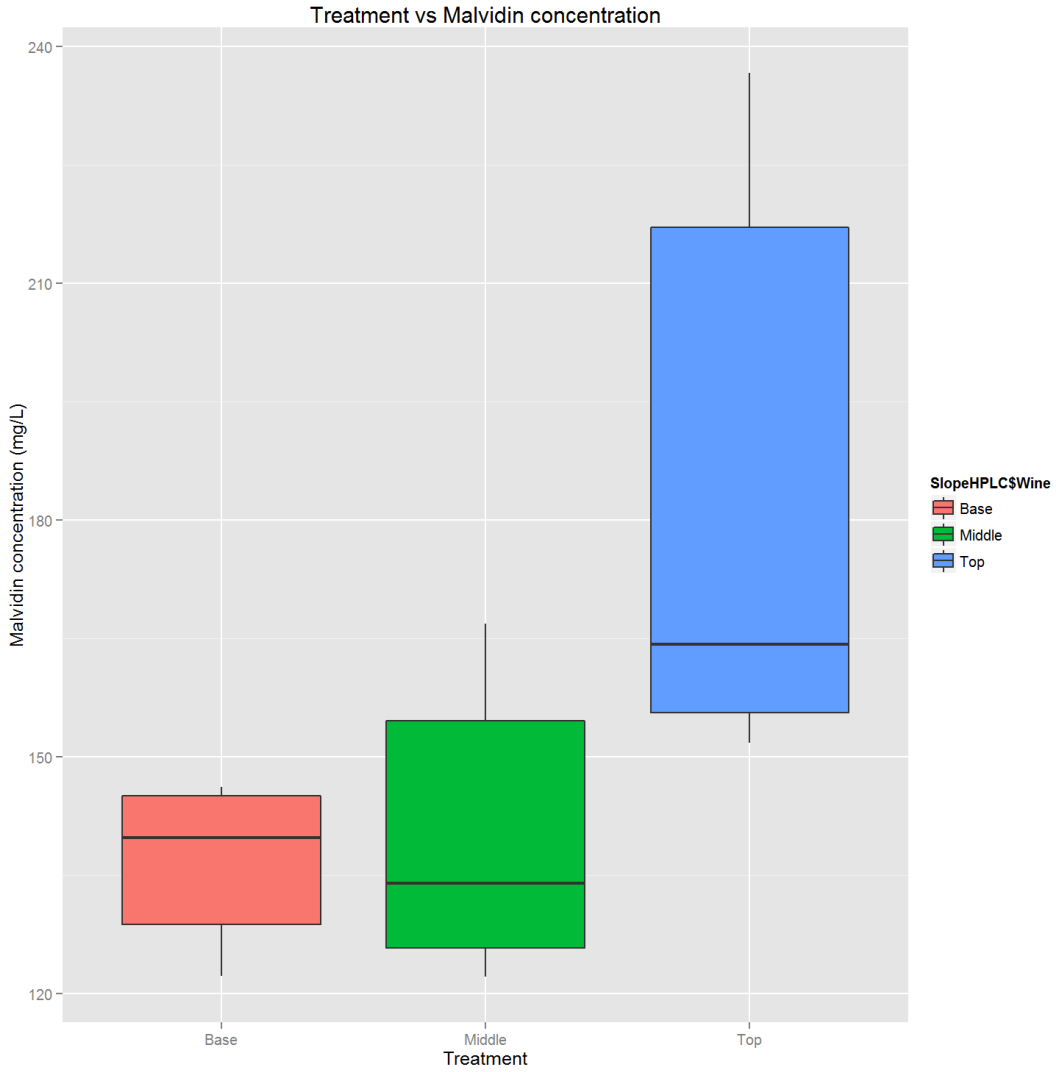


Figure 3.2: HPLC results for malvidin-3-glucoside concentrations versus slope position. Three replicate ferments were produced for each treatment, and two samples were read per treatment.

The suggestion that the position of the vines on the slope can influence wine character is not unprecedented: differences in aspect, soil type, depth, and soil moisture (which can vary across or up the slope) are known to influence wine character, as may any temperature gradient (Nicholas et al. 2011; Seguin 1986; Wittendal 2004). However, the results presented here demonstrate that minor differences in growing environment may not be enough to impact significantly

impact the wine quality factors measured in this study, with the exception of malvidin-3-glucoside concentration.

This study confirmed the utility of the Bodum press model for replicable very small scale ferments, agreeing with the conclusions of Dambergs et al. (2012). There was little variation between the ferments (with a coefficient of variation of less than 1 % for CIELab values for everything other than the a* and b* values of the wines from the top of the slope, which were 2.78 % and 3.32 % respectively). The ferments can be easily replicated, and the plunger mechanism allows for simple cap management and standardised pressure for skin separation.

Indeed, Dambergs et al. (2012) have suggested that multiple Bodum presses could be used to produce Bodum tank farms, allowing for easy-to-use, small scale, replicated ferments. Such tank farms could be used to assess the efficacy of new winemaking methods or additives, or differences between parcels of fruit, before scaling up to commercial production levels (Dambergs et al. 2012).

3.3.2 Whole bunch ferments compared with destemmed ferments

Figure 3.3 shows the twelve days of the fermentations, with pressing occurring on the twelfth day. Both the whole bunch and destemmed ferments proceeded smoothly and quickly, with no obvious problems such as sluggish or stuck fermentations observed. There were no discernible levels of hydrogen sulfide produced. No differences in fermentation dynamics between the ferments were noted. Note that sugars were not measured using refractometry below 3 °Bx as it is no longer a reliable method, and there was a slight delay before a more reliable method could be arranged. This did not affect the day on which the ferments were pressed, however.

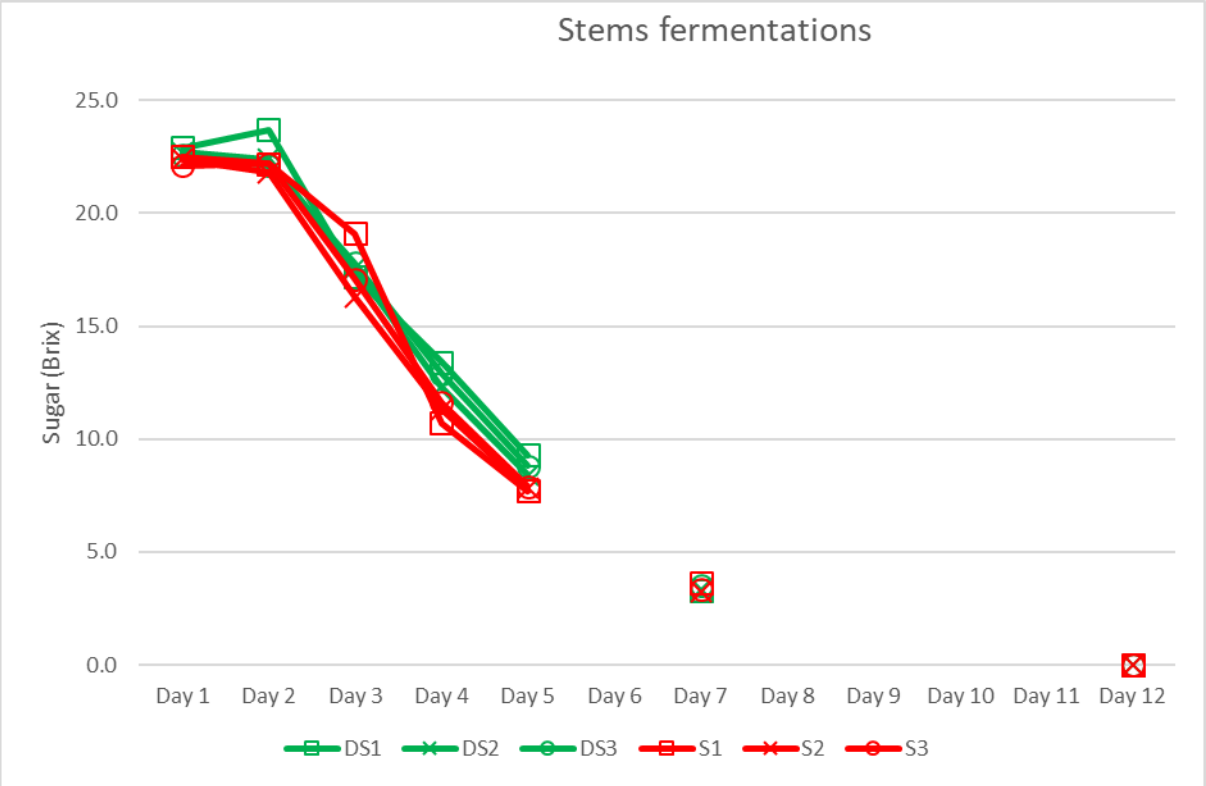


Figure 3.3: Fermentation graph of whole bunch vs destemmed ferments, showing reduction in sugar levels over the course of the fermentation.

Ferments: DS is destemmed, S is whole bunch. 1-3 represents the three replicates of each location.

Differences were observed between treatment and control in all CIELab components (see Table 3.1, Table 3.2, and Figure 3.4); these differences were particularly evident for b*, a result which the ANOVA analysis suggested was highly unlikely to have arisen by chance (F value of 91.39, *p* value of 5.12×10^{-8}). L* had an F value of 10.96 and a *p* value of 0.00441, while a* had an F value of 6.192 and a *p* value of 0.0182 (Appendix 2.3); while the differences between these were less obvious in the boxplots, nonetheless these results are significantly different. This predicts that Pinot Noir wines made with stems ferments will be measurably darker

in colour (lower L* values), and more magenta (higher a* values) and yellow (higher b* values), i.e. more 'brick red', than those made without stems.

That CIELab b* was much higher for whole bunch wines, confirming that wines fermented with stems were visibly yellower than those fermented without stems, is not surprising. It has been shown previously that wines fermented in contact with the stems show higher levels of tannins (Butler 1981; Dambergs et al. 2012; de la Barra et al. 2005; Goode 2012; Sun et al. 1999), and it is possible that this caused the colour difference noted. de la Barra et al. (2005) noted that wines fermented in contact with stems were darker in colour than the control, but no increase in yellow colour was noted. Sun et al. (1999) found that the stems added flavan-3-ols to the wine, which provide colour stability against sulfur dioxide bleaching and pH variation.

Table 3.1 - CIELab average values and standard deviations of whole bunch vs destemmed wines

Wine	L, Average	L, StdDev	a, Average	a, StdDev	b, Average	b, StdDev
Whole bunch	90.0	1.0	8.4	1.0	5.5	0.3
Destemmed	91.6	1.0	7.2	0.9	4.1	0.3

Table 3.2 -- Sommers Analysis values and standard deviations of whole bunch vs destemmed wines

Wines	Total anthocyanins mg/L, Average	Total anthocyanins mg/L, StdDev	Colour density au, Average	Colour density au, StdDev	Total phenolics au, Average	Total phenolics au,StdDev
Whole bunch	82.1	6.4	2.8	0.1	35.3	2.6
Destemmed	90.4	2.0	2.4	0.3	22.3	0.6

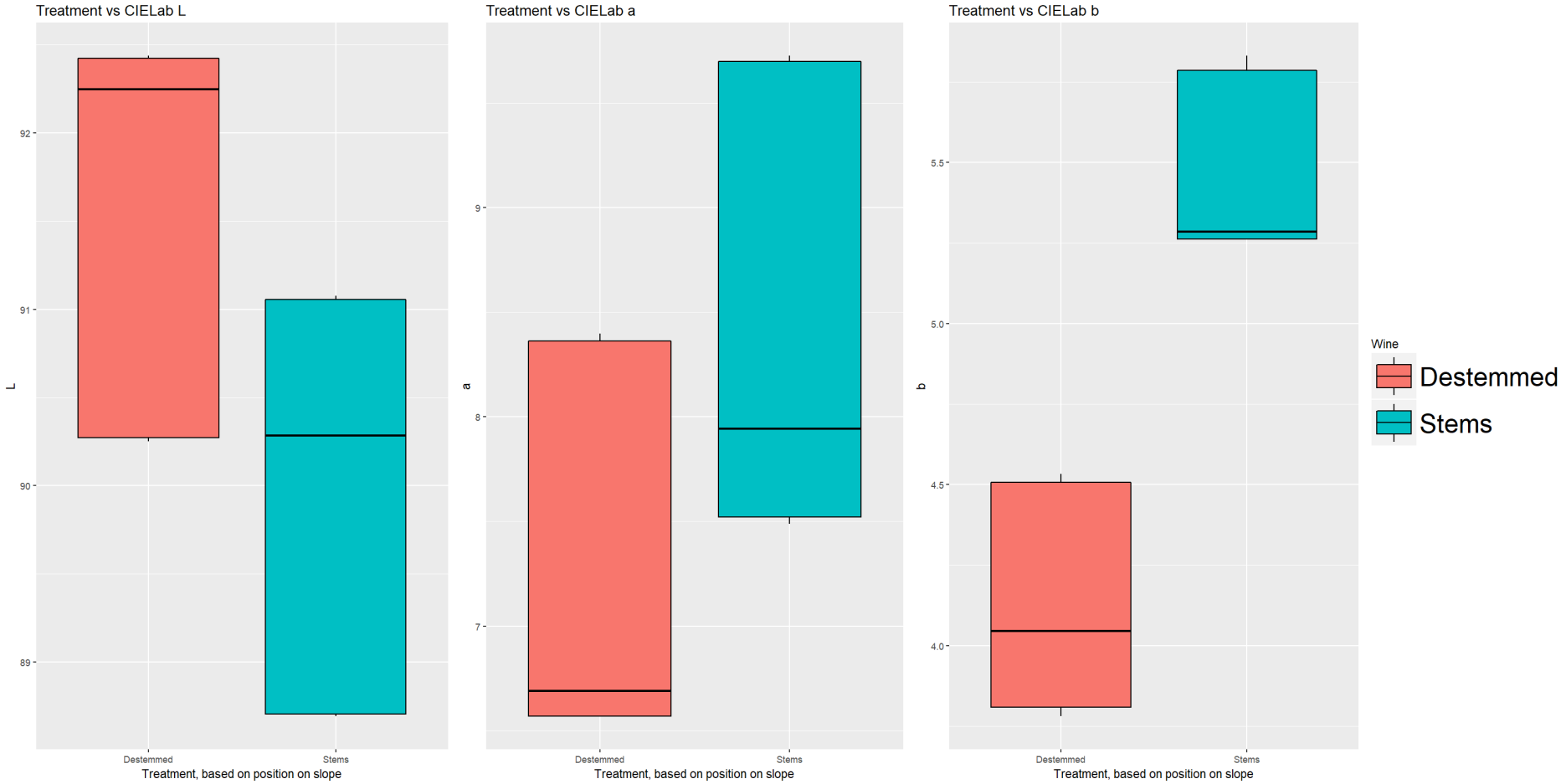


Figure 3.4: Box plots for CIELab parameters. Each replicate wine was sampled once, and each sample was read three times.

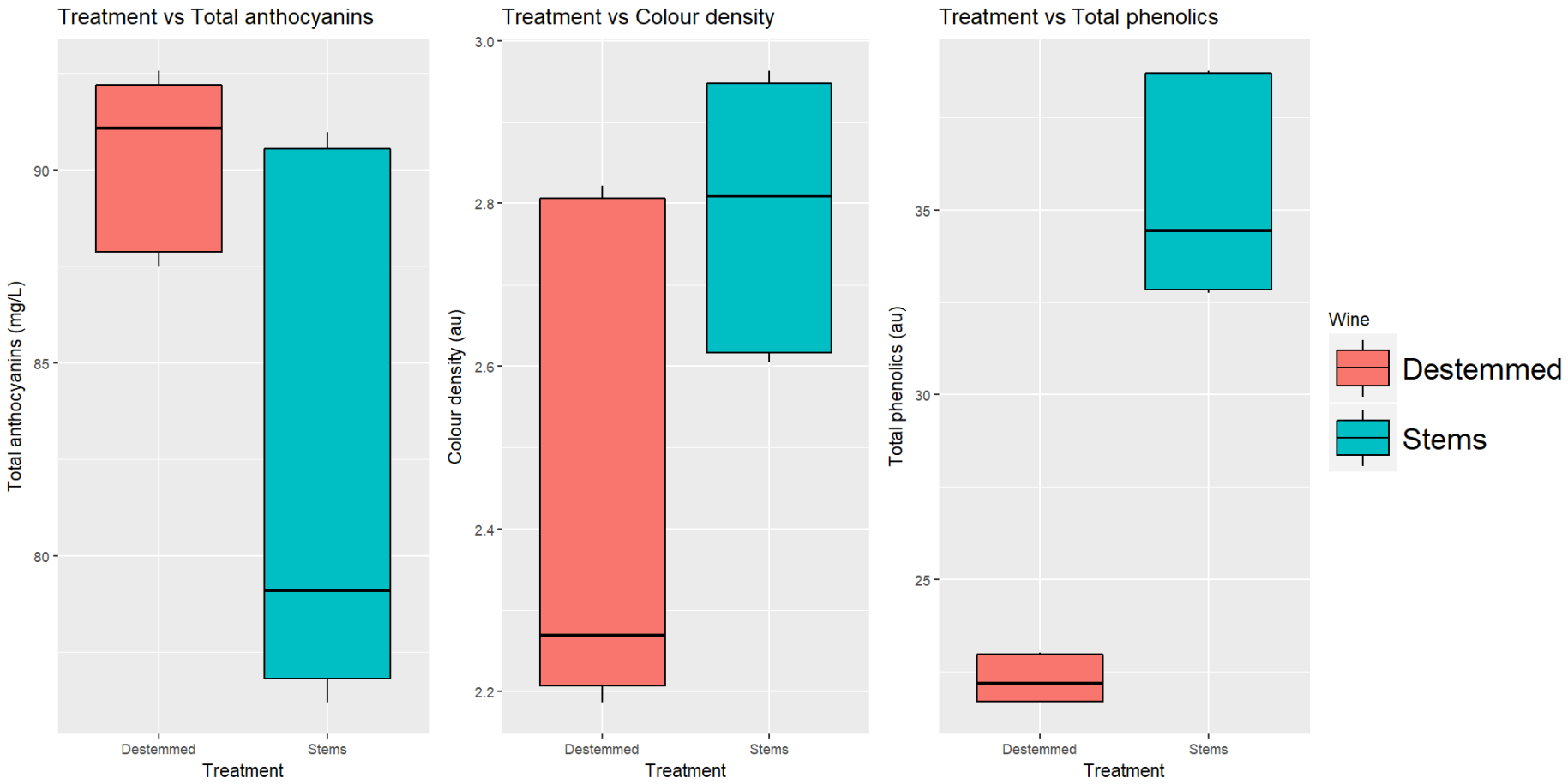


Figure 3.5: Box plots of results of the Somers' Methods analysis. Only a slight difference in anthocyanin levels or colour density between destemmed and stem ferments is observed. However, higher levels of phenolics are observed in ferments with stems included. Each replicate wine was sampled three times, and each sample was read once.

There were highly significant differences for all variables determined using the Somers' Methods, despite apparent overlapping in the box plots for Total Anthocyanins and Colour Density (and the relatively high standard deviation for Total Anthocyanins, see Table 3.2). The ANOVA tables showed clear differences between the treatments for all variables measured by Somers' Methods. Total Anthocyanins had an F value of 27.74 and a p value of 7.75×10^{-6}, Colour Density an F value of 23.90 and a p value of 2.43×10^{-5}, and Total Phenolics an F value of 443 and a p value of $<2.2\times10^{-16}$ (Appendix 2.4). These highly significant differences were less obvious in the box plots (see Figure 3.5), which showed considerable variability in the data with noticeable overlap for analyses other than Total Phenolics.

Wines made with stems showed lower total anthocyanin values, perhaps because anthocyanins were bound to tannins in these wines, or had been lost by adsorption on to the stems (Boulton 2001, p. 82). That the inclusion of stems in the ferment can increase Total Phenolics was not surprising: stems contain large amounts of tannins (Kantz & Singleton 1991; Souquet et al. 2000; Sun et al. 1999) that can be easily extracted during a fermentation. A previous study noted that addition of stems increased tannin levels significantly, albeit not pigmented tannins (Dambergs et al. 2012). It is interesting to observe that this appears to have a direct impact on colour, though this small scale study was unable to determine the precise mechanism: further investigation could be warranted. It is possible that the tannins could form copigmentation complexes with the anthocyanins, creating more permanent, stable compounds, and thus more stable wine colour (Butler 1981; Goode 2012; Gutiérrez, Lorenzo & Espinosa 2005; Souquet et al. 2000); however, the evidence is still inconclusive and further study is required (Boulton 2001; Pascual et al. 2016).

Consequently, it would be interesting to assess the exact impact of stem inclusion on colour, aroma, and flavour as the wine aged. Little research seems to have been done in this area, so it is difficult to predict the impact exactly. Henri Jayer, the Burgundian winemaker, claimed that destemming "doesn't affect the ageing of the wine" (Rigaux 2009, p. 124), although this opinion was the anecdotal experience of only one (revered and knowledgeable) *vigneron* rather the results of a scientific study. It is, however, possible that the addition of stems, and the resulting higher levels of tannins, may be beneficial for the production of polymeric coloured compounds, which are more stable and tend to persist in wine longer than simple, reactive anthocyanins. Such changes in wine chemistry during ageing have been discussed in Chapter 1 and by, for example, Brouillard, Chassaing and Fougerousse (2003); Brouillard and Dangles (1994); Parley, Vanhanen and Heatherbell (2001); & Revilla, I et al. (1999), but these authors did not discuss the impact of stem inclusion.

It is worth noting that inclusion of stems in fermentations also impacts on aroma and flavour, which have not been explored in this study. Henri Jayer, for example, claimed that including the stems would "only bring astringent, acerbic tannins" (Rigaux 2009, p. 124). In contrast, Tom Carson, winemaker at Yabby Lake (Tuerong, Mornington Peninsula, Vic, Australia), commented,

> "*When it's good, whole-bunch fermentation gives fragrance and perfume, and adds a bit of strength and firmness to the tannins. But when it's not good, it can dull the fruit, adding mulch and compost character*" (Goode 2012, p. 92).

Depending on stem ripeness (i.e., whether stems are brown and lignified *versus* green and unripe), terroir, grape character, and fermentation technique, this impact can either be positive or negative. Negative impacts include addition of overtly

'green'/herbal flavours, and an increase in wine pH. Positive impacts include increased flavour and aroma complexity (including savoury characters), and increased tannin levels (Goode 2012). This could also be of interest for future research, and it would be interesting to examine the impact of the amount of stem addition and the degree of lignification on wine colour and character.

3.4 Conclusions

3.4.1 Bodum press fermentation trial

The results showed that the location of vines on the slope, which can be considered as a micro-environmental parameter, may not be a confounding factor for any study of growing temperature effects on the colour and anthocyanin content of grape berries and wines. CIELab parameter b*, 'blueness/yellowness', showed a trend towards increasing up the slope, although this was not significant. Analysis of malvidin-3-glucoside also showed a trend towards increasing up the slope, which was significant for this prime red pigment. Given that the perceived colour (i.e., CIELab parameters) is a gross measure of overall pigment balance (including other pigments such as anthocyanins and polymeric pigments), it is not surprising that the contribution of malvidin to wine colour can be masked to some extent by other factors, such as other extractable pigments present in the berries.

It therefore seems appropriate to suggest that the position of the vine on a slope does not influence the perceived wine colour significantly, although it may be a contributing factor for a study of specific colour pigments.

The utility of the Bodum presses for very small scale, replicated ferments was confirmed, with reliable, steady fermentations occurring in all of the Bodum presses. The Bodum presses themselves are therefore recommended for small scale ferments,

because multiple small fermentations can be easily managed, and their plungers allow for replicable cap management and pressing. It is also beneficial that they are readily available and inexpensive.

3.4.2 Whole bunch ferments compared with destemmed ferments

The addition of stems to ferments had a significant impact on the finished wine, both in terms of colour and phenolics. Wines made with stems included in the fermentations were darker in colour (lower L* value, higher colour density), more magenta in colour (higher a* value), and more yellow (higher b*), this combination yielding a more intense 'brick red'. Stems yielded increased total phenolics and lower total anthocyanins.

This suggests that the addition of stems to fermentations may be beneficial, although additional study would need to be undertaken to ensure that aroma and flavour were not negatively impacted by use of stems. The impact on the colour of the wine as it ages may well be significant, however there is currently no published research to indicate precisely what the impact would be. It is suggested that this could be potentially of interest for future research. Future research could also examine the impact of the amount of stem addition (i.e., the percentage of whole bunches used) and the degree of lignification on wine character.

Chapter 4. Regionality in Australian Pinot Noir wines: a study on the use of NMR, ICP-MS, and Spectroscopy on commercial wines

Statement of Authorship

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Publication Status	☐ Published ☐ Accepted for Publication ☒ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
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Principal Author

Name of Principal Author (Candidate)	Gavin Duley		
Contribution to the Paper	I wrote the bulk of the paper, based on an initial idea suggested by Prof Dennis Taylor. After the retirement of Prof Taylor, I organised collaboration with colleagues at the Institut Heidger (Germany) and the Université de Bourgogne/AgroSup Dijon. I also ran statistical analyses, based on the advice of my colleagues in Dijon. My colleagues in Dijon also assisted with writing the paper, as discussed below.		
Overall percentage (%)	70%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	21 January 2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Signature		Date	December 5, 2019

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Contribution to the Paper	NMR analysis		
Signature		Date	16.12.2019
Name of Co-Author	Anna Werwein		
Contribution to the Paper	ICP analysis		
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Contribution to the Paper	NMR analysis		
Signature		Date	16.12.2019

Regionality in Australian Pinot Noir wines: a study on the use of NMR, ICP-MS, and Spectroscopy on commercial wines

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Abstract

Wine quality and character are defined in part by the *terroir* (climate, geology, aspect, phenology, cultural management techniques, and soils) in which the grapes are grown. Metabolomic techniques such as nuclear magnetic resonance (NMR) spectroscopy and inductively coupled plasma mass spectrometry (ICP-MS) have been used to characterise wines and to detect wine fraud in other countries, but their use has not been extensively trialled in Australia. This paper describes the use of ICP-MS and NMR to characterise the distinctiveness of a selection of Pinot Noir wines from southern Australian wine regions including varied geology, and both cool and warm climates. Central Otago (New Zealand) was included to act as an out-group. This study showed that the wines from varying *terroirs* can be somewhat distinguished by their mineral content using principal component analysis (PCA) and cluster analysis of ICP-MS data. In particular, cluster analysis defined seven clear clusters, but these did not align with region, state, or vintage. PCA was able to separate wines by their Australian states or origin more clearly than by climate or region. It is suggested that this is in part because Australian wine regions are quite large compared to Europe, and have heterogenous soils and geologies. Metabolomic analysis of the wines using NMR did not find any correlations with climate in terms of daytime temperatures. An analysis of coinertia suggested that the two datasets were not redundant, and it is proposed that ICP-MS data is the most useful for determining regionality.

Keywords: NMR; ICP-MS; Pinot Noir wine; terroir; metabolomics

Declarations of interest: none

Novelty statement: This paper is the first to use highly sensitive metabolomic techniques to examine regionality in Australian Pinot Noir wines, and the first to use a combination of ICP-MS and NMR with PCA to analyse regionality in Australian wines. It also compares the usefulness of the two techniques.

4.1 Introduction

4.1.1 Terroir

The environment in which the grapes are grown has an impact on the characteristics of the grapes and wine[2]. In particular, Roullier-Gall, Boutegrabet, Gougeon, and Schmitt-Kopplin [1] drew attention to the environmental effects on the metabolic pattern (or 'metabolome') of a wine:

> *"Wine can be described as a molecular kaleidoscope integrating signatures from grapes, yeasts, the winemaking and the environment, reflected in the variety of compounds, including primary (e.g., sugars, organic acids, amino acids) and secondary metabolites (e.g., flavonoids, anthocyanins, and other pigments)."* [1] (p. 100)

Greenough, Longerich, and Jackson [2] suggest that it is possible that "wine element 'fingerprints' are relict soil signatures that survive metabolic and winery processing" [2] (p 75). Indeed, it has been pointed out that any particular wine "arises from a complex interplay between environmental, genetic and human factors" [3] (p. 1). In this study, the environmental and geographic factors—which form part of what is referred to as '*terroir*' [1, 3-11]—are primarily of interest, where terroir can be defined as, "*an interactive ecosystem, in a given place, including climate, soil, and the vine (rootstock and cultivar)*" [Seguin 1988, cited in 9] (p. 207).

[2] List of abbreviations. FTICR-MS: Fourier-transform ion cyclotron resonance mass spectrometry; HPLC: High performance liquid chromatography; ICP-MS: inductively coupled plasma mass spectrometry; NMR: nuclear magnetic resonance; MFA: Multifactorial analysis, MJT: Mean July/January Temperatures (note that January is used in the Southern Hemisphere and July in the Northern Hemisphere), PCA: Principal component analysis, NOESY: Nuclear Overhauser Effect SpectroscopY, PGI: Protected Geographical Indication.

Elsewhere, Roullier-Gall, Boutegrabet, Gougeon, and Schmitt-Kopplin [1] spoke of terroir as:

> "*an usually rather small area whose soil and microclimate impart distinctive qualities to food products [...] and it is now accepted that metabolites in wine could be affected by the terroir", and that "terroir is the greatest determining factor of all great wines*" [1] (pp. 100-101).

Barnard [11] remarked on the links between the words 'terroir' and 'territory', and that

> "*This territory possesses certain homogeneous physical properties (soil, geology, drainage, etc.) which are suitable for certain agricultural products. Specifically, the nature of the soil is thought to be communicated through the character of certain products, most notably with wine. Wine, according to its terroir, will have a particular taste that is associated with the soil, geology, topography, and climate where the vineyard is located.*" [11] (pp. 14-15)

More simply, terroir can be thought of as, "the taste of the place" [11] (p. 15), although Bessis, Fournioux, and Jeandet [12] (p. 129) have noted that arguments continue about "*the alchemy of typicity*."

4.1.2 Nuclear Magnetic Resonance spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is a powerful chemical analytical technique that has been used frequently to identify organic compounds in complex mixtures, such as wine [13]. Metabolic patterns ('metabolomics') have been used to attempt to chemically define grape varieties, regionality, and vintages [14,15], and as a tool for determining authenticity and traceability of European wines [16, 17]. In

particular, NMR has been used to determine the geographical area of production (including climate and soil) [6, 16, 18], vintage effects [19], variety [19, 20], and yeast strain [6].

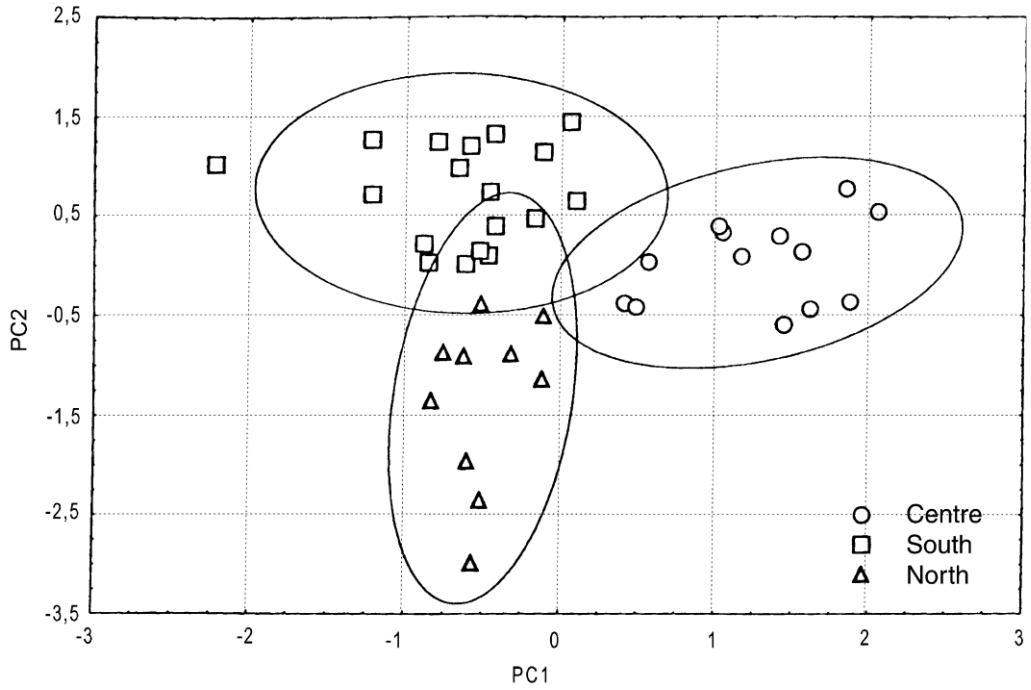


Figure 4.1: Scatter plot of two principle component analyses of 1H NMR analytical data, from Brescia, Caldarola, De Giglio, Benedetti, Fanizzi, and Sacco [14] (p. 181)

NMR has become a powerful tool to detect wine fraud, including chaptalisation or addition of water, as well as misrepresentation of wine region of origin, where wine from a less prestigious appellation is sold as being from a more prestigious (and hence more expensive) appellation [13-15, 18, 21-24]. Studies have used both stable isotope NMR and, more recently, metabolomic methods. In these studies, wines from the same region are shown to form distinct clusters via statistical analysis. For example, Figure 4.1 shows how principal component analysis (PCA) of data from ^{1}H NMR analysis provided statistical separation of viticultural regions, in

this case the north, south and central areas of Apulia, southern Italy) [14] (p. 181). NMR has also been demonstrated to easily detect variations between vintages within a region [13, 21, 24].

Godelmann, Fang, Humpfer, Schütz, Bansbach, Schäfer, and Spraul [15] proposed a number of methods that might be of interest, including a number of different ^{1}H NMR methods. ^{2}H and ^{13}C fingerprinting of fermentative ethanol [21], or 1D ^{1}H and ^{13}C spectra [22] might be of use for determining regionality at a small scale, including vine response to environmental stress (e.g., excessive heat) [21]. Additionally, $\delta^{13}C$ and $^{2}H/^{1}H$ values (both defined by Giménez-Miralles, Salazar, and Solana [21] (p. 2646)) determined from fermentable ethanol can also be of use for determining regionality [21].

4.1.3 Inductively coupled plasma mass spectrometry (ICP-MS)

ICP-MS has also been used to determine regionality in wine by building up a 'fingerprint' based on chemical composition of trace elements that are present in wine, some of which are considered to derive from vineyard soils [2, 25-30]. As Frías, Conde, Rodríguez-Bencomo, García-Montelongo and Pérez-Trujillo remarked, "minerals are the best placed to perform a differentiation according to the geographical origin due to the direct relationship with soil composition" [31] (p. 336), as it provides greater sensitivity and selectivity [32].

ICP-MS is of interest in determining wine fraud, where wine produced from one appellation is passed off as the produce of another. Other analytical methods used to produce a 'chemical fingerprint' of a wine include stable isotope analysis, inductively coupled plasma atomic/optical emission spectroscopy (ICP-AES/OES), and related

techniques [17, 26, 33, 34]. However, particularly in some of European wine regions, their close proximity makes chemical discrimination legally challenging:

> "*Although fingerprinting approaches are powerful, they are also harder to standardize across labs. Certification processes and databases for certain measurements have been developed – particularly isotope ratio measurements – but the uncertain robustness of other promising fingerprinting approaches in the literature has limited their widespread adoption*" [17] (p. 390).

A number of studies have been undertaken, for example, employing ICP-MS to distinguish Canadian wines from two distinct regions (Okanagan Valley and Niagara Peninsula) [27]. A similar study in the Czech Republic, where both the wines and soils from six viticultural regions were analysed using ICP-MS, showed that the soil and wine characteristics clustered well by region, with soil samples clustering better than wine samples [25]. Rocha, Pinto, Almeida, and Fernandes [35] used flame atomic absorption spectrometry and ICP-MS to differentiate wines from different Portuguese Protected Geographical Indications, while Pérez-Álvarez, Garcia, Barrulas, Dias, Cabrita, and Garde-Cerdán [32] used ICP-MS to distinguish wines from different growing zones within the Rioja *denominación de origen calificada* (DOCa). The precise elements that were considered useful varied based on the regions being studied, and were particular to each study.

Other mass spectrometry techniques, such as Fourier-transform ion cyclotron resonance mass spectrometry (FTICR-MS), have been used with notable success [1, 3]. This technique has also shown promise in distinguishing wine vintages, as well as vineyards [1].

4.1.4 Terroir and Australian wine regions

Since wine regions in Australia are mostly defined by political boundaries rather than geologically defined boundaries, the ranges of soils and geologies within a region such as the Adelaide Hills may be very heterogenous [36, 37]. This makes it a somewhat harder task to chemically define a wine as having been grown in a specific region. Thus, Stern, and Fund [37] noted that

> "*when you consider the diversity of grape varieties, viticultural practices and vinification methods used in any particular region as well as the youth in wine terms and the sheer size of many of the regions, it is clear that in such circumstances, it is difficult if not impossible to say that there can be any 'typicite'*[sic] *or identifiable 'terroir' of our regions.*" [37] (p. 41)

However, it can be argued that Australian regions are still in the process of identifying their *terroirs*, and of defining *typicité*, and it is likely that—as part of this process—smaller subregions will be defined, similar to Europe. In Australia, for example, the Adelaide Hills already has subregions defined within them (Lenswood and Piccadilly Valley) [38, 39].

Nonetheless, a few metabolomic studies have already been undertaken to assess regionality in Australian wines. Simpler analytical technologies such as spectroscopy have been studied to distinguish between wines from Australia, New Zealand, and Europe, although no attempt was made to distinguish between regions within these countries [40, 41]. Spectroscopy has been used to distinguish between Sauvignon blanc wines from New Zealand and Australia, again without

distinguishing between regions within either country [42]. High performance liquid chromatography (HPLC) with UV and chemiluminescence detection was able to distinguish between wines from the Coonawarra and Geelong districts[43]. Riovanto, Cynkar, Berzaghi, and Cozzolino [5] used spectroscopy to distinguish between Shiraz wines from the large state of West Australia and the South Australian district of Coonawarra, but it was less able to distinguish between other South Australian regions.

Importantly, Martin, Watling, and Lee [26] used profiles of metallic ions, determined by ICP-MS to distinguish between various Australian wine regions, but also noted difficulty in differentiating some South Australian wine districts, perhaps due to their close proximity or similar soil types. Gambetta, Cozzolino, Bastian, and Jeffery [44] used Headspace Solid Phase Microextraction-Gas Chromatography-Mass Spectrometry, Gas Chromatography-Mass Spectrometry, Inductively Couple Plasma-Optical Emission Spectroscopy, and HPLC to determine regionality in Chardonnay berries sampled from several regions across South Australia. More recently, Šuklje, Carlin, Antalick, Blackman, Deloire, Vrhovsek, and Schmidtke [45] used two-dimensional gas chromatography coupled to time-of-flight mass spectrometry to distinguish Shiraz wines from the Orange and Riverina regions of New South Wales. In particular, this study noted that wines from the cooler climate region of Orange were higher in compounds such as monoterpenes and sesquiterpenes, a difference which would presumably be changeable with vintages.

4.1.5 Aims

This study aimed to determine whether bottled wines from different regions in Australia can be distinguished using metabolomic methods (NMR and ICP-MS) and

chemometrics. Similar methods have been used for other agricultural products to determine growing region or cultivar, including olive oil [46], corn steep liquor [47], and wine vinegar [48].

4.2 Methods

4.2.1 Selection of wine samples

Bottled vintage wines were donated or purchased from a number of regions across Australia, with Central Otago, New Zealand, used as an out-group (Table 4.1). The geographic separation of most areas was very large by European standards, so the selection included both cool and warm climates. The wineries were contacted for further details, such as whether the wines contained grapes other than Pinot Noir (15% of other varieties can be included for a varietally labelled wine without being declared on the label), the soil type, and whether the wine had undergone malolactic fermentation. Where provided, this information is also shown in Table 4.1, along with the Mean January/July Temperatures (MJTs; note that January is used in the Southern Hemisphere and July in the Northern Hemisphere, for the peak Summer month) of the regions included in this study [49-55].

4.2.2 Lab analyses

Analyses of wine colour and sulfur dioxide (sulfite) content were performed in the Food and Wine laboratories of the School of Agriculture, Food and Wine at The University of Adelaide (Urrbrae, South Australia, Australia). Spectrophotometric analyses were performed using a Cintra 4040 UV/Visible spectrophotometer (GBC Scientific Equipment, Melbourne, Australia), with CIELab [56, 57] and Somers Analysis undertaken. Each sample was read twice. A narrow (1 mm path length)

quartz cuvette was used for the CIELab analysis. Somers Analysis was undertaken to determine the total phenolics, total anthocyanins, and colour density of the experimental wines [58-61], as per the protocol of Mercurio, Dambergs, Herderich, and Smith [58], but with only total phenolics, total anthocyanins, and colour density measured. Sulfur dioxide was measured using the Aspiration Method, using the method proposed by Iland, Bruer, Edwards, Weeks, and Wilkes [62].

4.2.3 NMR and ICP-MS (Institut Heidger)

In this study, NMR and ICP-MS were used to determine whether the regional differences between the regions investigated produces demonstrable differences in the finished wines. This has been shown in other regions, e.g. Rioja [16].

NMR and ICP-MS were undertaken at the Institut Heidger (Osann-Monzel, Germany), according to their validated proprietary methods. NMR was performed using an Agilent 600 MHz NMR spectrometer (Agilent, Santa Clara, CA, USA). The pulse sequence utilised NOESY (Nuclear Overhauser Effect SpectroscopY)-first increments, with presaturation of background water and ethanol signals. Quantitative analysis was undertaken using MNova (Mestrelab, Santiago de Compostela, Spain). The NMR analyses were focused on specific groups of metabolites, rather than generalised scans to gather a 'metabolome'. The metabolites studied included amino acids, sugars, acids, alcohols, pigments, and their precursors. These are influenced by both the grapes and the winemaking procedures, such as fermentation parameters, time of skins in must, use of pH adjustment, and secondary fermentation (malolactic conversion).

ICP-MS was undertaken using an Agilent 7700 x ICP-MS, following the Heidger Institute's proprietary methods. The analyses measured the following

groups of inorganic anions: light metals (e.g. sodium, Na; aluminium, Al; etc); transitional/heavy metals (e.g. copper, Cu; lead, Pb); rare earth elements (e.g. ytterbium, Yb; dysprosium, Dy; praseodymium, Pr); and non-metallic elements (e.g. arsenic, As).

These may derive from the vineyard soil minerals and underlying geology, as well as previous mining activity in the area. However, they can also be influenced by vineyard management, including dissolved minerals in irrigation water if used and fungicides (such as copper salts), and by winery processing methods, including the use of metal pipes and tanks (e.g. trace iron, chromium, or aluminium contamination).

4.2.4 Multivariate data analysis

PCA was undertaken, following the example of previous authors [29, 64], using the 'dudi.pca' function of the 'ade4' package in early trials [65-67] and the 'PCA' function of the 'FactoMineR' package for published data [68]. Data were normalised using the scale argument of the 'dudi.pca' or 'FactoMineR' functions. Factors that were invariant or only had one measurement were removed from the analysis (Appendix 3.3). An exploratory analysis with PCA was conducted. Outliers were detected (ICP-MS: YV04, AH02, & AH06 and NMR: CO-04; a rationale for their exclusion is provided in the Results and discussion section) and removed for the following analyses. These were determined to be outliers based on visual inspection of the PCA biplots (see, for example, Figure 4.2 a & b and Figure 4.5 a. b, & c), with individuals that were distant from the main cluster of individuals were considered to be outliers. Hierarchical clustering analysis was undertaken to produce dendrograms using the 'hcut' function of the 'factoextra' package [69] and the 'hclust' function of the 'stats'

package. All analyses were undertaken using RStudio version 1.1.453 with GNU R version 3.6.1 (2019-07-05) [70, 71].

To evaluate the contributions of the NMR and ICP-MS data, a multifactorial analysis (MFA) and an analysis of coinertia were undertaken. MFA analysed the described observations in several "blocks" or sets of variables, seeking the common structures present in all or some of these sets [72]. This was undertaken using the 'MFA' function of the 'FactoMineR' [68] and 'factoextra' packages [69], and the analysis of coinertia was undertaken using the 'dudi.pca' and 'coinertia' functions of the 'ade4' package [65-67]. Analysis of coinertia explores the covariance between two datasets, by finding a structure or co-structure to examine the relationship between two set of variables where the two datasets are linked by the same individuals [73] Both of these analyses were undertaken using RStudio version 1.1.453 with GNU R version 3.6.1 (2019-07-05) [70, 71]. In addition, multidimensional scaling and t-distributed stochastic neighbour embedding were trialled, but not used in the final analysis.

Table 4.1: Wine properties

Wine code	Region	Vintage	State/Country	MJT	Alcohol, ABV (label claim)	Irrigation source	Geology	Mining	100% Pinot	Varieties	MLF
AH-01	Adelaide Hills	2010	SA	20.4 °C [49]	13.5%	Bore	(NR)	No	(NR)	(NR)	(NR)
AH-02	Adelaide Hills	2012	SA	20.4 °C [49]	13.5%	Dam	Sandy clay loam topsoil between 10 and 30 cm, orangey clay subsoil between 10 and 120cm. Shaley weathered rock underneath, hardening as it deepens	Yes, mainly gold, unsure if on property	Yes	PN	Yes
AH-03	Adelaide Hills	2012	SA	20.4 °C [49]	12.5%	Bore and dam	Acid loam over red clay on siltstone	No	Yes	PN	Yes
AH-04	Adelaide Hills	2012	SA	20.4 °C [49]	13.5%	(NR)	(NR)	(NR)	(NR)	(NR)	(NR)
AH-05	Adelaide Hills	2011	SA	20.4 °C [49]	14.1%	Bore (one vineyard with high salinity & high iron content)	Site A: gentle slope valley floor with dark alluvial clay or young river bed grey clay; Site B: Hills Face Loamy topsoil to gravelly yellow clay and shale with alluvial clay to deep motley yellow clay; Site C: Sloping Hillside Loamy grey top soil, alluvial red yellow	No	Yes	PN	Yes

							clay with ironstone gravel, and deep alluvial clay				
AH-06	Adelaide Hills	2013	SA	20.4 °C [49]	14.0%	(NR)	(NR)	(NR)	(NR)	(NR)	(NR)
CO-01	Central Otago (NZ)	2013	NZ	18 °C [74]	13.0%	(NR)	(NR)	(NR)	(NR)	(NR)	(NR)
CO-02	Central Otago (NZ)	2013	NZ	18 °C [74]	13.0%	Bore & rivers	Schist and mount wash from glaciers	Gold (19th century)	(NR)	(NR)	(NR)
CO-03	Central Otago (NZ)	2012	NZ	18 °C [74]	13.5%	Bore	Loam over gravel, schist bedrock	Gold panning, no heavy machinery	Yes	PN	Yes
CO-04	Central Otago (NZ)	2011	NZ	18 °C [74]	13.5%	Spring or river-fed dam	Schist with varying levels of topsoil	Gold mining, but not on the vineyard sites		(NR)	(NR)
MP-01	Mornington Peninsula	2011	VIC	19.3 °C [50]	13.0%	Dam (but not in 2011 due to high rainfall)	Most vineyards on basalt clay, with approx. 35% on old sandstone-based soil	No	No	PN + 1-2% Shiraz	(NR)
MP-02	Mornington Peninsula	2010	VIC	19.3 °C [50]	14.0%	Three vineyards: non-irrigated; dam; grey water from sewage	Sandy loam on clay	No	Yes	PN	Yes

						treatment plant					
MP-03	Mornington Peninsula	2012	VIC	19.3 °C [50]	13.5%	Bore and dam	Fairly typical soil from the Northern growing areas of Mornington	No	Yes	PN	Yes
MP-04	Mornington Peninsula	2012	VIC	19.3 °C [50]	13.0%	(NR)	Two sites on red volcanic basalt, the other one sandy grey loams	No	Yes	PN	Yes
MP-05	Mornington Peninsula	2013	VIC	19.3 °C [50]	13.5%	Non-irrigated	Ferrasol or sedimentary brown loam	No (quarrying w/in 20km)	Yes	PN	Yes
MV-01	McLaren Vale	2012	SA	21.3 °C [51]	13.5%	Bore	Biscay/Sandy soil	No	Yes	PN	Yes
MV-02	McLaren Vale	2012	SA	21.3 °C [51]	14.0%	Bore	Biscay/Sandy soil	No	Yes	PN	Yes
MV-03	McLaren Vale	2012	SA	21.3 °C [51]	14.0%	Bore	Urbrea silt loam, Christies beach formation	No	Yes	PN	Yes
SF-01	Southern Fleurieu	2010	SA	20 °C [52]	13.0%	Dam	Ironstone ridge running through the vineyard		Yes	PN	Yes
SF-02	Southern Fleurieu	2010	SA	20 °C [52]	13.3%	Dam prior to 2005, then irrigated	(NR)	(NR)	Yes	PN	Yes
SF-03	Southern Fleurieu	2012	SA	20 °C [52]	14.0%	Dam	The soil is a quite acid duplex soil which we	yes, in the region but	Yes	PN	Yes

							have heavily limed. It is a free draining sandy loam over kaolinite clay with a metamorphised sand stone/ironstone parent material	not near the vineyard			
TAS-01	Tasmania	2011	TAS	15.6 °C [53]	13.6%	Dam	Site A: black vertosols, brown demosols, black self-mulching clay, over rolling basalt and dolerite hills Site B: duplex soils with yellow to grey clay subsoils and coarse quartz sand, loamy sand, and heavy ironstone clay loam topsoils	No	Yes	PN	Yes
TAS-02	Tasmania	2013	TAS	15.6 °C [53]	14.0%	Dam	Site A: highly duplex soils with a clay subsoil and topsoils from sandy loam to loam Site B: black vertosols, brown demosols, black self-mulching clay, over rolling basalt and dolerite hills Site C: duplex soils with yellow to grey clay subsoils and coarse quartz sand, loamy sand, and heavy ironstone clay loam topsoils	No	No	PN, 2% Sauvignon blanc for yeast propagation	Yes

TAS-03	Tasmania	2012	TAS	15.6 °C [53]	13.5%	Dam	Dolorite, perhaps with ironstone aggregate	Unsure	Yes	PN	Yes
TAS-04	Tasmania	2012	TAS	15.6 °C [53]	13.7%	(NR)	(NR)	(NR)	(NR)	(NR)	(NR)
TAS-05	Tasmania	2013	TAS	15.6 °C [53]	13.4%	(NR)	(NR)	(NR)	(NR)	(NR)	(NR)
TAS-06	Tasmania	2013	TAS	15.6 °C [53]	13.9%	60% river, 40% dam	From sedimentary river flats to shallow dolerite	No	Yes	PN	Yes
YV-01	Yarra Valley	2012	VIC	18.9 °C [54]	13.5%	Dam	Brown loam over friable clay	No	Yes	PN	Yes
YV-02	Yarra Valley	2010	VIC	18.9 °C [54]	13.5%	Dam, topped up with bore	Fine, silty clay	(NR)	Yes	PN	Yes
YV-03	Yarra Valley	2012	VIC	18.9 °C [54]	13.2%	(NR)	(NR)	(NR)	(NR)	(NR)	(NR)
YV-04	Yarra Valley	2013	VIC	18.9 °C [54]	13.0%	Mostly dam or creek	Mostly from vineyards with sandy loam, some from basaltic or volcanic soils	No	No	> 85% PN + other varieties	Yes

Note – MJT is 'Mean January/July Temperature', with the temperature recorded in mid-summer, i.e. January in the southern hemisphere, or July in the northern hemisphere. MJTs for all Australian regions are from 1991-2017. For comparison, Burgundy has an MJT of 19.7 and Bordeaux an MJT of 20.3 [55]. (NR): the winery representative did not respond with relevant information; NZ: New Zealand; SA: South Australia (state), VIC: Victoria (state); TAS: Tasmania (state); PN: Pinot noir.

4.3 Results and discussion

4.3.1 Lab analyses

As expected, the parameters from the university lab analyses (wine colour intensity and sulfur dioxide) primarily reflect viticultural and winemaking decisions, not regionality. The results can be seen in Table A1 of Appendix 3.1.

4.3.2 ICP-MS data

An initial PCA showed that several variables were highly correlated, including most of the rare earth elements, which all correlated with each other, and thorium, which correlated with bismuth, tungsten, germanium, and vanadium. In addition, three outlier vineyards were found to have a disproportionate influence on the model (see Figure 4.2 a & b). These were:

- AH-02, characterised by high values of thulium, lutetium, holmium, lanthanum, terbium, erbium, ytterbium, dysprosium, praseodymium, and gadolinium (in descending order of importance)
- AH-06, characterised by high values of rubidium, thallium, and caesium (in descending order of importance)
- YV-04, characterised by high values of tungsten, bismuth, thorium, germanium, vanadium, arsenic, cerium, samarium, and zirconium (in descending order of importance).

The wines from these 'outlier' vineyards can be hypothesised to indicate the presence of particular mineral deposits, and thus they may represent extreme values that may typify some geological aspects of outcrops or washouts in some areas. With

respect to AH-02, the 'heavy' rare earths, including neodymium, praseodymium, terbium, and dysprosium, are in high demand for permanent magnets used in renewable energy, electric vehicles, personal technology, and robotics, and virtually all of the heavy rare earth element deposits in Australia are found in inland fossil beach strandlines. There are a large number of such sites to the east and north of the Adelaide Hills region, such as the Mindarie Sands [75]. Similarly, regarding AH-06, the close association of rubidium and thallium in some minerals, including the caesium mineral pollucite, has been noted previously [76], and these 'alkali elements' are typical in 'australites' deposits found in some areas of South Australia. With respect to YV-04 in Victoria, thorium is frequently associated with rare earth elements, for example in the mineral monazite, and historically Australia has exported large quantities of monazite from heavy mineral sands mined in Western Australia, New South Wales, and Queensland. Smaller scale extraction has occurred in a variety of locations, but interestingly none is reported in Victoria, however it can be speculated that the YV-04 vineyard soil may possibly contain, for example, alluvial washouts of monazite [77].

In the absence of more complete data on these more extreme geologies, the analysis was rerun without these wines (see Figure 4.2 c & d). In this analysis, the remaining wines were more clearly separated, and this allowed the relationships between all wines to be more clearly observed.

The first two PCs explained 44.24% of total inertia (i.e., 44.24% of total variability was provided by the PC1-PC2 plane). With four PCs, 64.13% of total inertia was explained, which allowed for improved interpretation. In particular, PC1 (see Figure 4.2 e) opposed the wines MP-01, MP-03, MP-04, and SF-03 with extremely negative coordinates (on the left), with MV-01, MV-02, and SF-02 having

extremely positive coordinates (on the right). The element dysprosium correlated strongly (correlation=0.91) with PC1, and could summarise this dimension by itself. PC2 (see Figure 4.2 e) placed wines such as TAS-01, TAS-02, and TAS-03 (on the top), in opposition to MP-01, MP-03, MP-04, MP-05, and SF-03 (at the bottom). PC3 (see Figure 4.2 f) opposed wines such as SF-01, AH-04, TAS-06, and TAS-01 (on the right), to CO-04, CO-03, and MV-03 (on the left). PC4 (see Figure 4.2 f) opposed SF-01 and SF-02 (on the right), to MV-01, CO-03, and CO-04 (on the left). Figure 4.2 e & f also illustrates the usefulness of the rare earth metals in the analysis.

Figure 4.3 shows the contribution of ICP-MS variables to the analysis. The importance of the rare earth elements was also immediately apparent, but some other metallic and non-metallic elements such as sodium, arsenic, and vanadium were clearly also of interest. In general, the elements found in the wine can result from vineyard soil and geology, but may be influenced by from winemaking and viticultural techniques. These can include irrigation water (dam or river water versus groundwater from bores or wells; groundwater can be a source of Na and As) [78], viticultural treatments such as Bordeaux mixture (Cu) [33, 34], fining agents such as bentonite (Cu, Fe, Mn) [79], and winemaking equipment, e.g. stainless steel tanks (Fe), tap water (for cleaning), and fittings (Al, Cu) [2, 27, 33, 63]. Heavy metal contamination of soils can be a result of past mining activity in the region [78]. However, metal levels can change during fermentation, as yeasts can remove copper from the must. In contrast, yeast cannot remove iron from the must [79].

In addition, the wines were categorised into groups using confidence ellipses (see Figure 4.2 e, f, g) to show ‘clustering’, similar to published NMR and ICP-MS data. Figure 4.2 e shows the wines grouped by region. While some regions overlapped considerably, several others were easily distinguishable. The South

Australian regions of Southern Fleurieu and McLaren Vale overlapped, but the two together formed a relatively distinct group, though there was some overlap with other regions (notably the South Australian Adelaide Hills region). This appears logical, as the Southern Fleurieu, McLaren Vale, and Adelaide Hills regions are contiguous.

Some regions were more difficult to distinguish, although Tasmania, Adelaide Hills, Yarra Valley, and Mornington Peninsula, which are widely separated in Australia, were all quite distinct from each other. However, McLaren Vale overlapped with the widely separated Adelaide Hills, Yarra Valley, and Central Otago (New Zealand), while Central Otago overlapped with diverse Australian regions of Tasmania, Adelaide Hills, McLaren Vale, and Yarra Valley. Interestingly, this suggests that the mineral contents of soils of the NZ Central Otago region are not radically different to those of the wines from the Australian winegrowing regions studied despite the radically different geological histories of Australia and New Zealand. Further research is needed to determine if this is true for other regions of New Zealand.

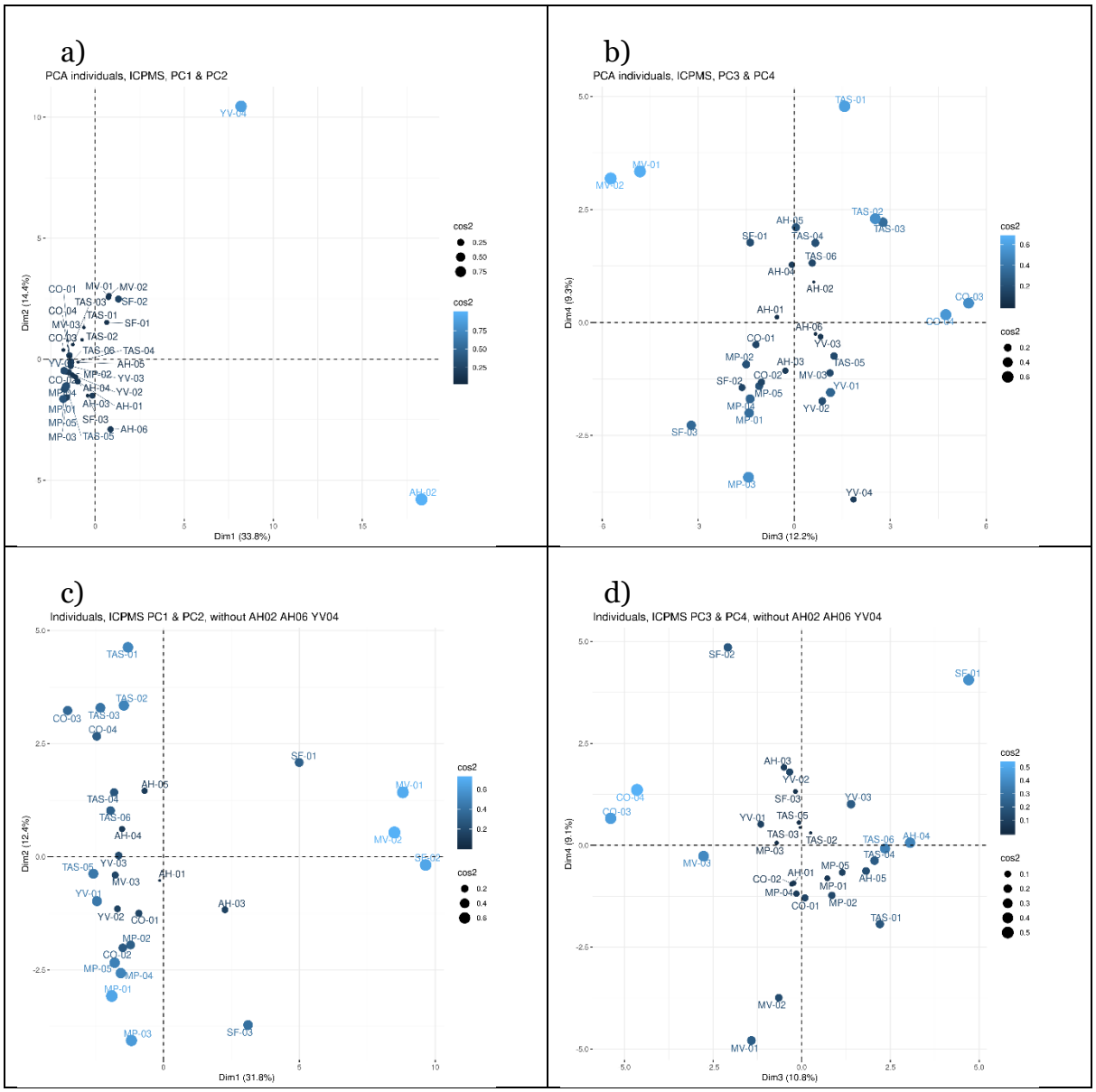
Figure 4.2 f shows the wines grouped by state. This confirmed that these political divisions had little relevance to the results. While the geographical isolation of South Australia appeared to be reasonably distinct in soil minerals, as seen in Figure 4.2 e, the states of Victoria and Tasmania overlapped with each other, and with New Zealand. However, Figure 4.2 g suggests that ICP-MS was not a useful method for distinguishing between vintages for the regions studied, as all four vintages (2010, 2011, 2012, 2013) clustered together.

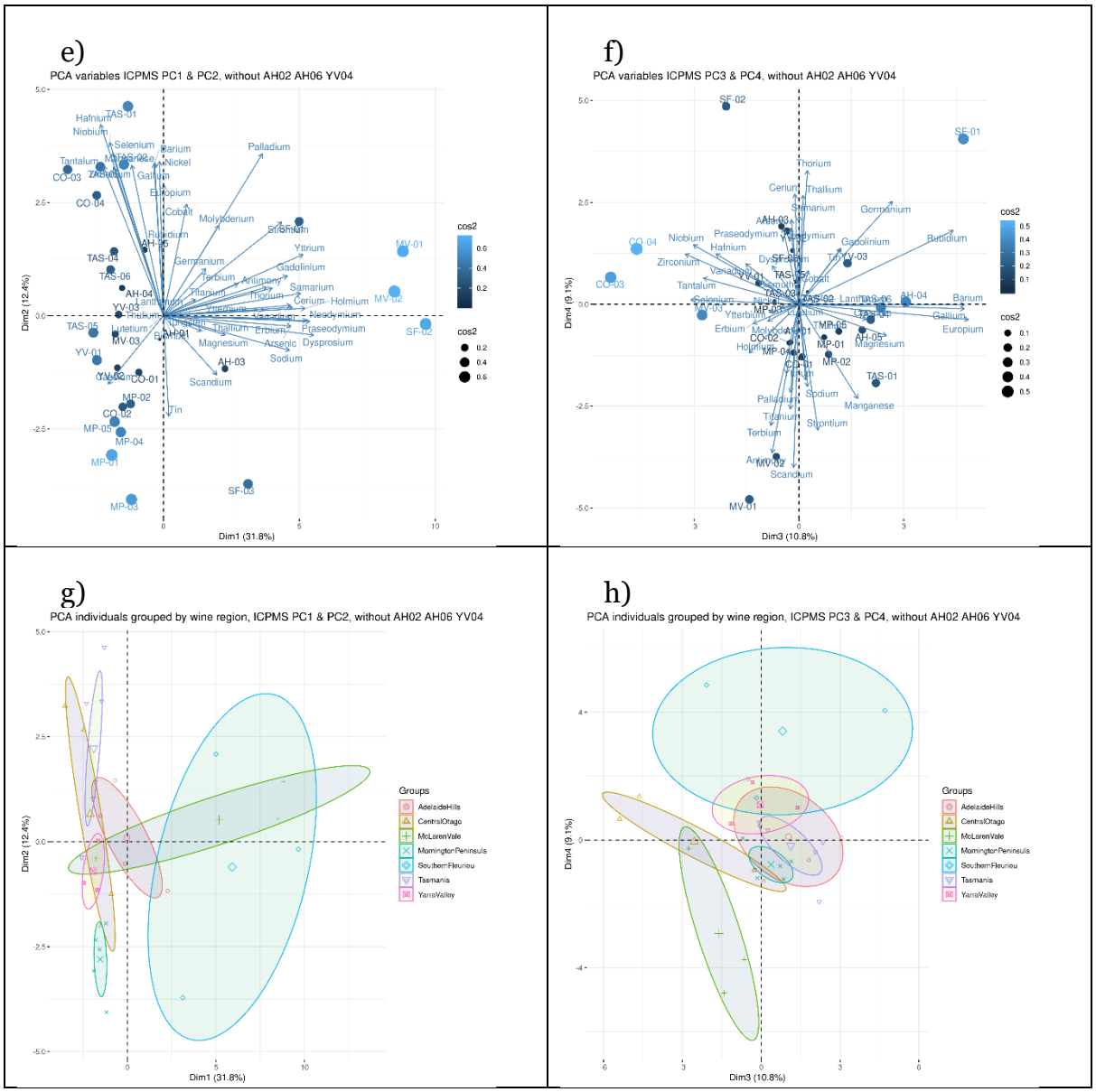
Finally, Figure 4.4 shows a dendrogram (a) and cluster diagram (b) of the the ICP-MS results for individual wines. There appeared to be seven distinct clusters, but

these did not neatly correlate with states or vintages, but showed some associations of geographical regions (Table 4.2). Cluster 1 consisted of CO-03 and CO-04; cluster 2 consisted of TAS-01, TAS-02, TAS-03, and others; Cluster 3 consisted of MP-01, MP-03, MP-04, MP-05, and others; cluster 4 principally consisted of SF-03; cluster 5 consisted solely of SF-01; cluster 6 consisted of MV-01 and MV-02; and cluster 7 consisted solely of SF-02. Noting the previous removal of outliers AH-02, AH-06, and YV-04, it was interesting that none of the AH nor YV wines were clustered.

Table 4.2: Clusters determined using cluster analysis, ICP-MS data without AH-02, AH-06, YV-04

Cluster	Composition	High values	Low values
1	CO-03 CO-04	Se, Zr, Hf, Nb, Ta	Rb, Mg
2	TAS-01 TAS-02 TAS-03	Ga, Ba, Rb, Eu, Mn, Hf	--
3	MP-01 MP-03 MP-05	Bi, Li, Tm	Pd, Sm, Sr, Ba, Nd, Y, Ga, Rb, Ce, Hf
4	SF-03	Sn, Mg, Nd, Sm, Yb	--
5	SF-012	Ge, Gd, Sm, Th, Ce, Ba	--
6	MV-01 MV-02	Sb, Sr, Y, Ho, Na, Pd, Tb, Er, Dy, Sc	--
7	SF-02	V, Th, As, Pr, Dy, Ce, Nd, Ho, Sm, Tl	--


a)
PCA individuals, ICPMS, PC1 & PC2
Dim1 (33.8%)
Dim2 (14.4%)
cos2
b)
PCA individuals, ICPMS, PC3 & PC4
Dim3 (12.2%)
Dim4 (9.3%)
cos2
c)
Individuals, ICPMS PC1 & PC2, without AH02 AH06 YV04
Dim1 (31.8%)
Dim2 (12.4%)
cos2
d)
Individuals, ICPMS PC3 & PC4, without AH02 AH06 YV04
Dim3 (10.8%)
Dim4 (9.1%)
cos2


e)
PCA variables ICPMS PC1 & PC2, without AH02 AH06 YV04
Dim1 (31.8%)
Dim2 (12.4%)
cos2
f)
PCA variables ICPMS PC3 & PC4, without AH02 AH06 YV04
Dim3 (10.8%)
Dim4 (9.1%)
cos2
g)
PCA individuals grouped by wine region, ICPMS PC1 & PC2, without AH02 AH06 YV04
Dim1 (31.8%)
Dim2 (12.4%)
Groups
AdelaideHills
CentralOtago
McLarenVale
MorningtonPeninsula
SouthernFleurieu
Tasmania
YarraValley
h)
PCA individuals grouped by wine region, ICPMS PC3 & PC4, without AH02 AH06 YV04
Dim3 (10.8%)
Dim4 (9.1%)
Groups
AdelaideHills
CentralOtago
McLarenVale
MorningtonPeninsula
SouthernFleurieu
Tasmania
YarraValley

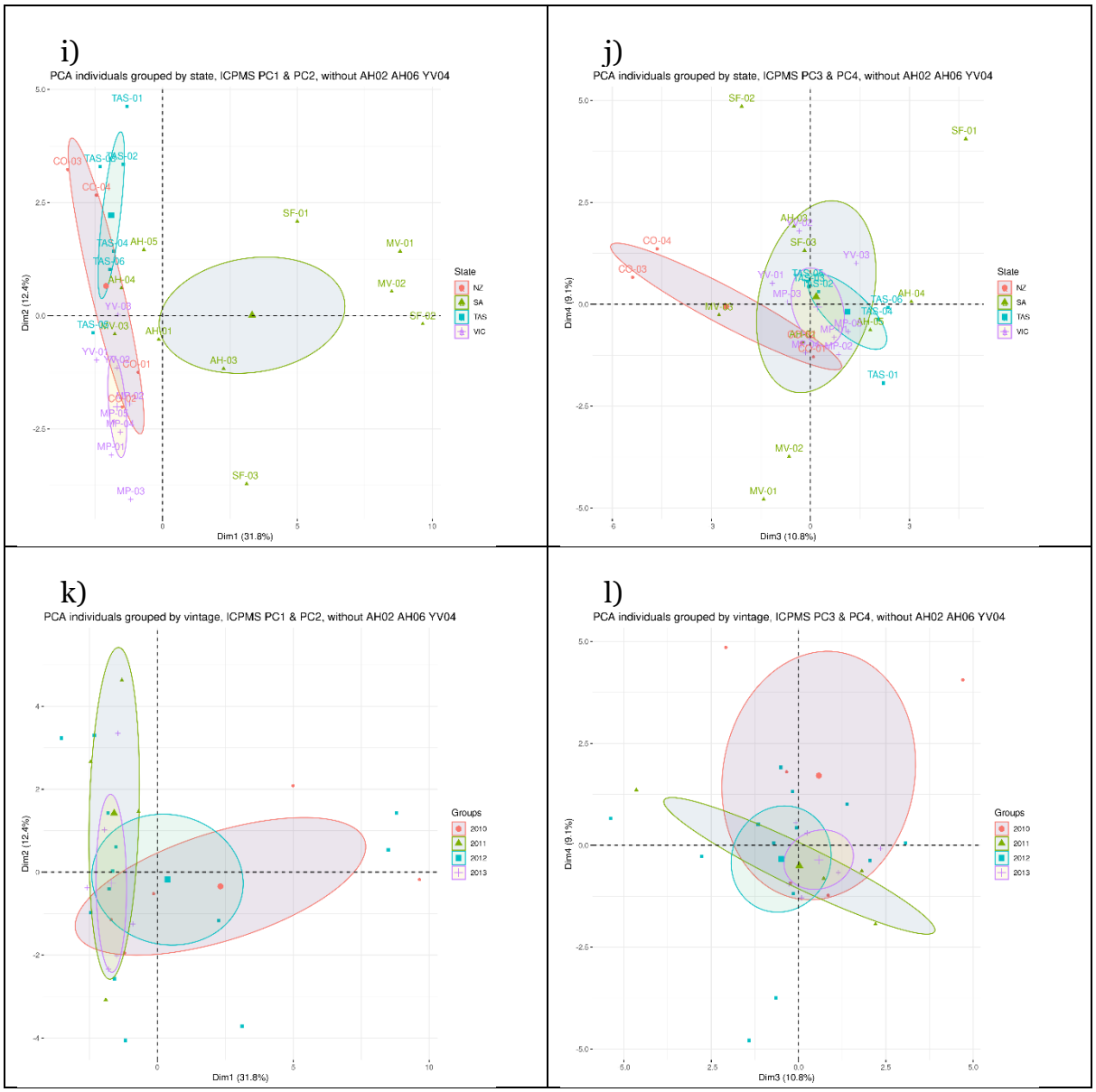


Figure 4.2: PCA of ICP-MS data

a-b) PCA, with extreme individuals (AH-02, AH-06, YV-04); c-d) PCA without extreme individuals, providing a clearer picture of their distribution within the PCA space; e-f) contribution of variables to PCA without extreme individuals; f-h) Individuals grouped by wine region using confidence ellipses, without extreme individuals; i-j) Individuals grouped by state using confidence ellipses, without extreme individuals (NZ: New Zealand; SA: South Australia; TAS: Tasmania, VIC: Victoria); k-l) Individuals grouped by vintage using confidence ellipses, without extreme individuals. The cos2 values (a-f) are used to estimate the quality of the representation. Variables with low cos2 values will be coloured white, variables with mid-cos2 values will be coloured blue, and variables with high cos2 values will be coloured in red. Point size was plotted according the cos2 of the corresponding individuals.

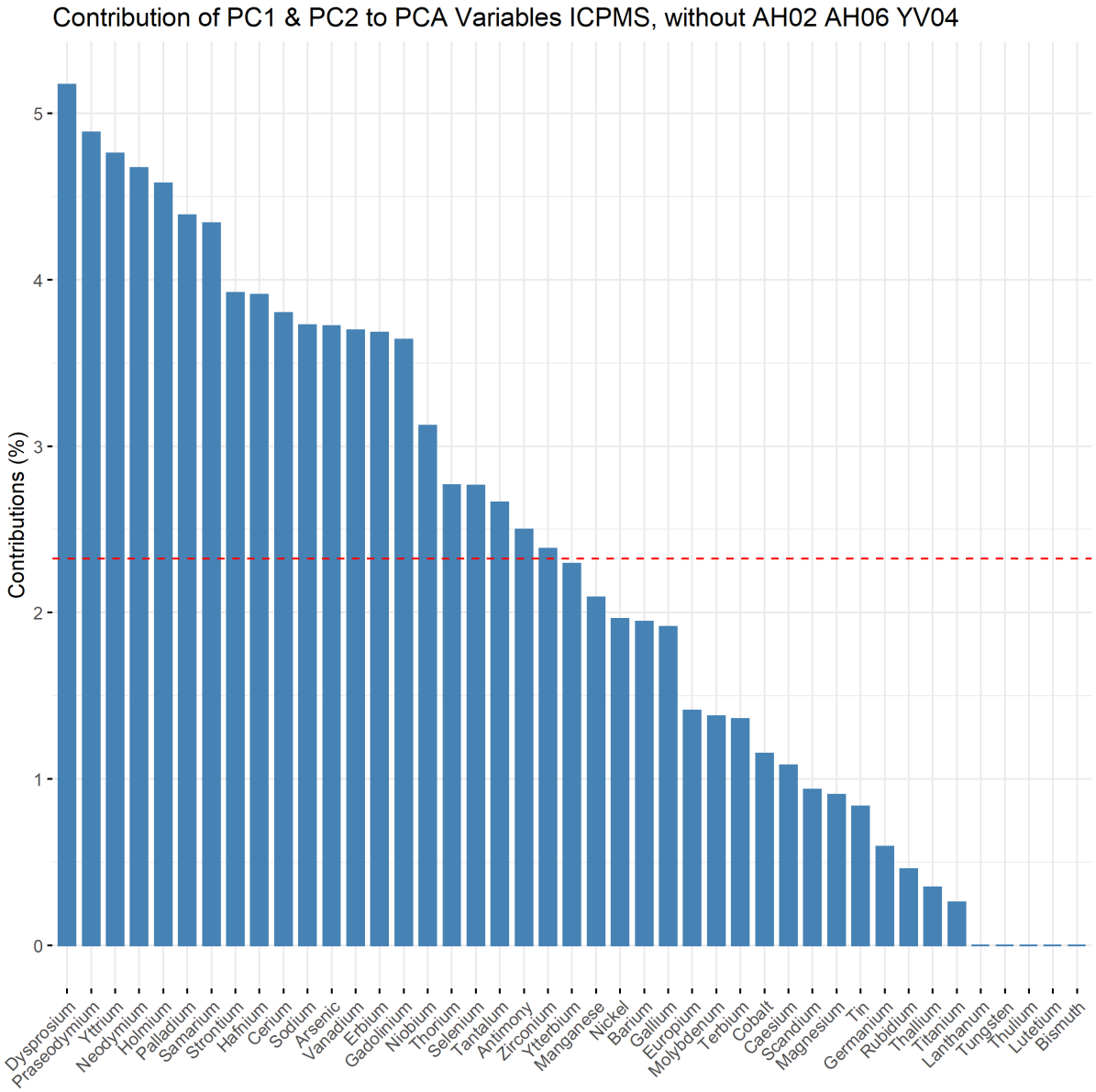


Figure 4.3: Contribution of ICP-MS variables to PC1 & PC2, without extreme samples.

The expected average contribution is indicated by a dashed red line.

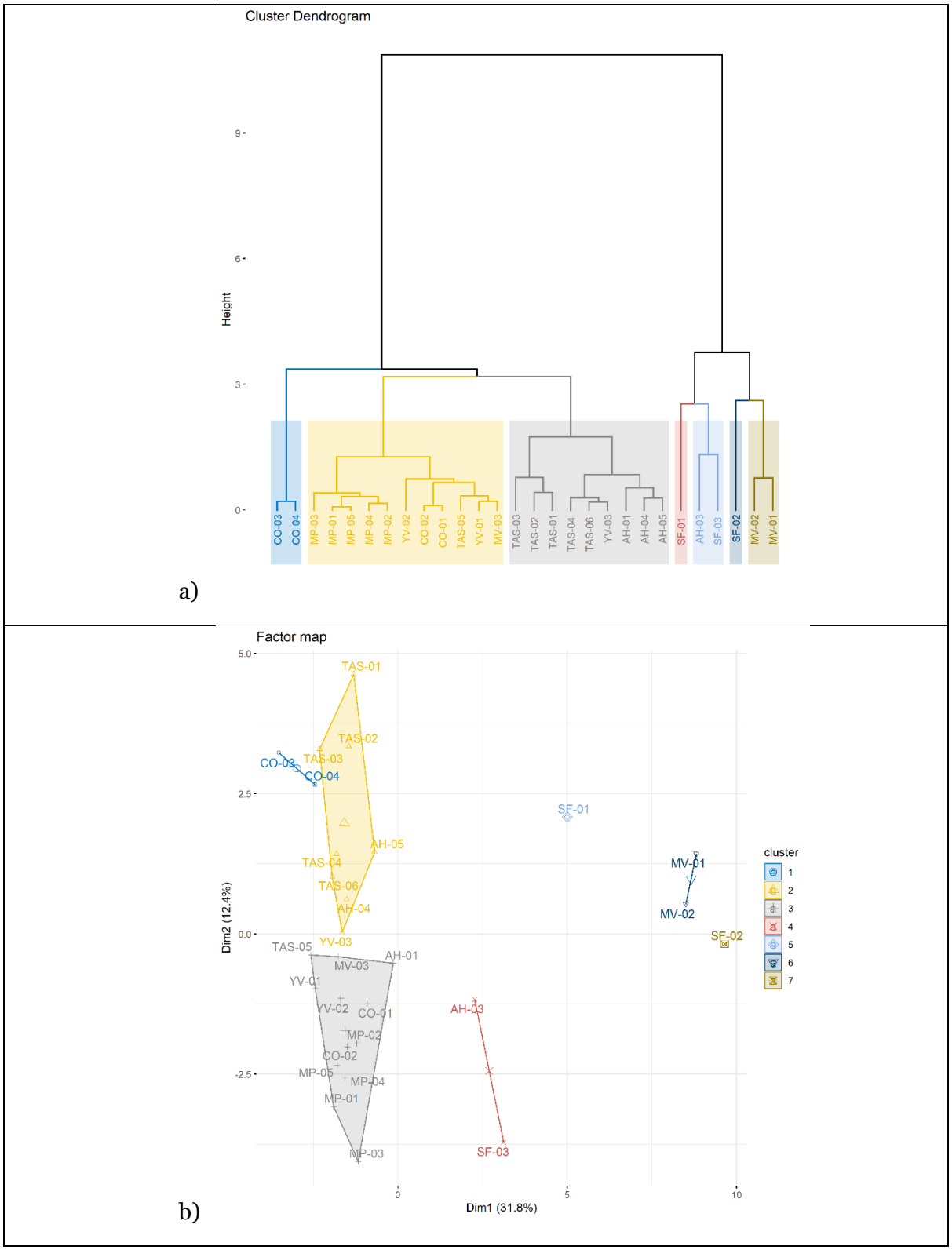


Figure 4.4: Clusters determined using cluster analysis

a) Dendrogram; b) Clusters shown in PC1 & PC2 space

4.3.3 NMR data

An initial analysis of wine organic metabolites by NMR showed that CO-04 had a disproportionate influence on the analysis, contributing 19.5% to the first plane (see Figure 4.5 a-c). It was characterised by high levels of the amino acids isoleucine, valine, threonine, leucine, and histidine, as well as 2-phenylethanol — an important flavouring with a pleasant floral scent. It was the only 2011 wine from Central Otago included in the study, and it is possible that the high levels of these compounds reflect that particular vintage (see also the more detailed discussion of vintage effects in the Multifactorial analysis sub-section, below).

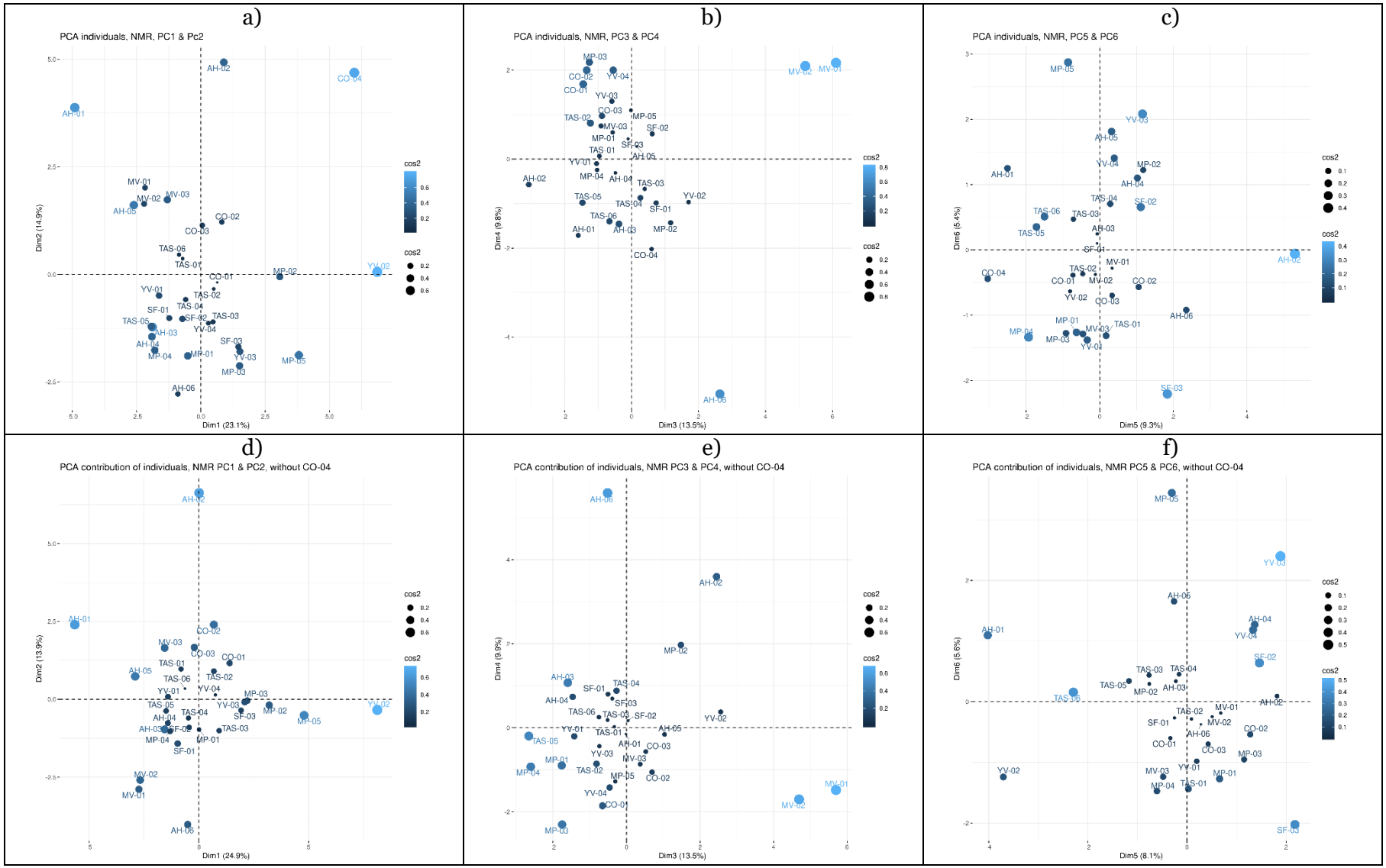
When the analysis was rerun excluding CO-04, valine, alanine, leucine, lactic acid, catechin, and succinic acid were all important variables in the PC1-PC2 plane (see Figure 4.5 d-i). Since the first two PCs explained only 38.86% of total inertia, 5 PCs were examined during the analysis; use of these 5 PCs provided 70.38% of inertia.

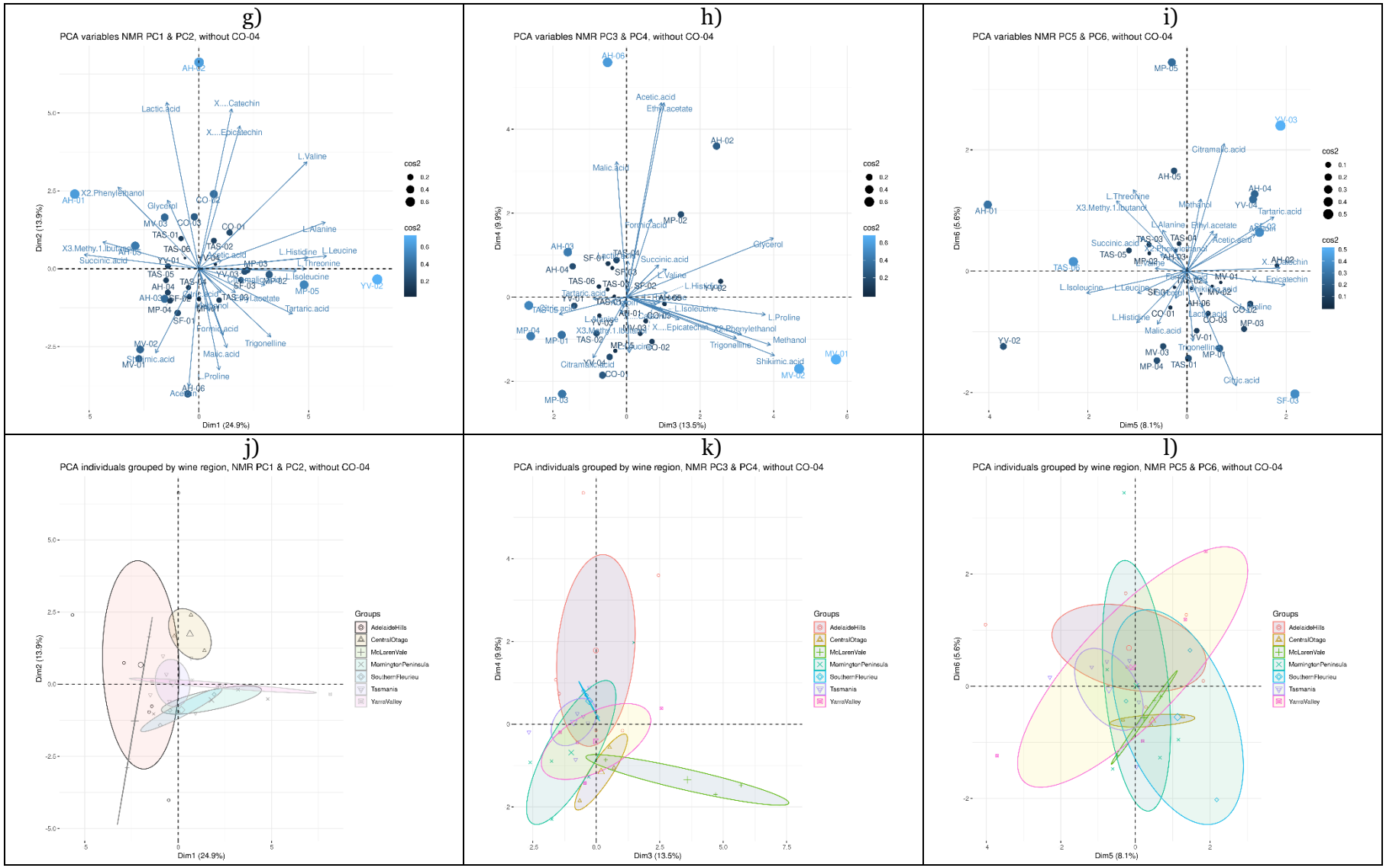
The different PCs were useful for separating out individual regions, based on different variables. PC1 distinguished YV-02, MP-05, and MP-02 (Figure 4.5 g). PC2 contrasted wines such as AH-01, AH-02, AH-05, and CO-02, against MV-01, MV-02, and AH-06 (Figure 4.5 g). PC3 contrasted individuals such as MV-01 and MV-02, compared to MP-04, MP-03, TAS-05, and CO-01 (Figure 4.5 h). PC4 was particularly useful for distinguishing certain individuals, such as AH-06 and AH-02 (Figure 4.5 h). PC5 contrasted wines such as YV-03, SF-02, MP-05, YV-04, and AH-04, in contrast to TAS-06, AH-01, and YV-02 (Figure 4.5 i).

The wines were also categorised into groups using confidence ellipses (see Figure 4.5 j-r), similarly to the ICP-MS and MFA data. Figure 4.5 j-l shows the wines

grouped by region. As expected, NMR metabolites did not prove particularly useful for distinguishing wines by region, and there was a lot of overlap between the groups. PC3 and PC4 (Figure 4.5 k), however, showed some differentiation between Adelaide Hills and McLaren Vale. The Adelaide Hills wines were defined by malic acid, acetic acid, ethyl acetate, and formic acid, while wines from the adjacent McLaren Vale was defined by shikimic acid, methanol, and trigonelline (see Figure 4.6).

Interestingly, there was a better separation based on Australian states (Figure 4.5 m-o), particularly in PC1&PC2 (Figure 4.5 m) and PC3&PC4 (Figure 4.5 o). This presumably reflected the differences in climates, which is quite marked between the three states studied. Central Otago, New Zealand, was well separated in both Figure 4.5 m and Figure 4.5 o, again presumably influenced by the far cooler climate. South Australia was also somewhat separated in Figure 4.5 o. As with ICP-MS, it was not possible to confidently distinguish between vintages, based on NMR data (Figure 4.5 p-r). A larger sample set is probably required. Cluster analysis was also used to create a dendrogram (Appendix 3.2, Figure A1 a) of the wines using the NMR data. Since this did not lead to meaningful categorisations, it is discussed briefly in Appendix 3.2.


a)
PCA individuals, NMR, PC1 & Pc2
Dim1 (23.1%)
Dim2 (14.9%)
b)
PCA individuals, NMR, PC3 & PC4
Dim3 (13.5%)
Dim4 (9.8%)
c)
PCA individuals, NMR, PC5 & PC6
Dim5 (9.3%)
Dim6 (5.4%)
d)
PCA contribution of individuals, NMR PC1 & PC2, without CO-04
Dim1 (24.9%)
Dim2 (13.9%)
e)
PCA contribution of individuals, NMR PC3 & PC4, without CO-04
Dim3 (13.5%)
Dim4 (9.9%)
f)
PCA contribution of individuals, NMR PC5 & PC6, without CO-04
Dim5 (8.1%)
Dim6 (5.6%)
cos2


g)
PCA variables NMR PC1 & PC2, without CO-04
Dim1 (24.9%)
Dim2 (13.9%)
h)
PCA variables NMR PC3 & PC4, without CO-04
Dim3 (13.5%)
Dim4 (9.9%)
i)
PCA variables NMR PC5 & PC6, without CO-04
Dim5 (8.1%)
Dim6 (5.6%)
j)
PCA individuals grouped by wine region, NMR PC1 & PC2, without CO-04
k)
PCA individuals grouped by wine region, NMR PC3 & PC4, without CO-04
l)
PCA individuals grouped by wine region, NMR PC5 & PC6, without CO-04
Groups
AdelaideHills
CentralOtago
McLarenVale
MorningtonPeninsula
SouthernFleurieu
Tasmania
YarraValley
cos2

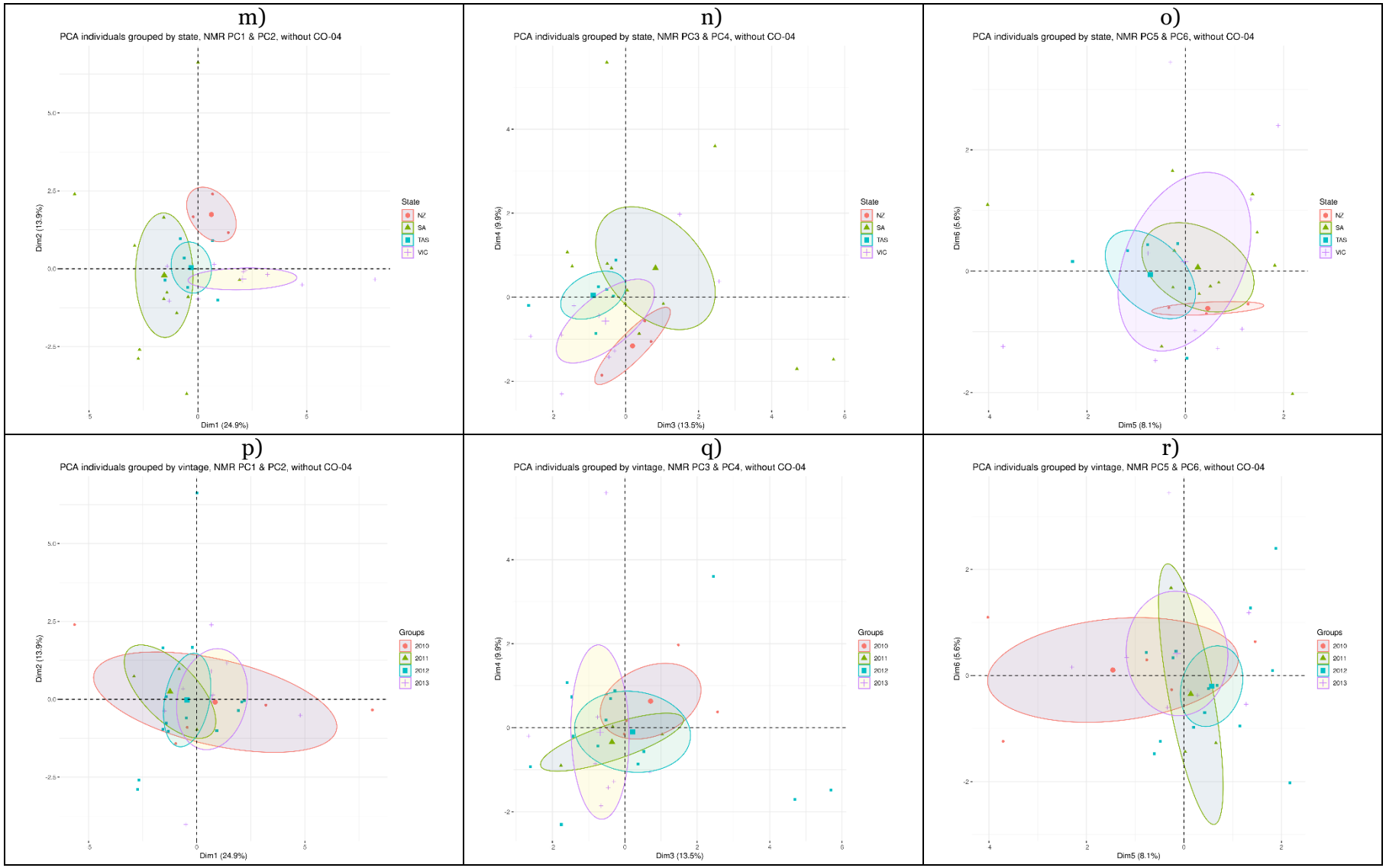


Figure 4.5: PCA of NMR data

a-c) PCA, with extreme individual (CO-04); d-f) PCA without extreme individual, providing a clearer picture of their distribution within the PCA space; d-f) Contribution of variables to PCA with CO-04; g-i) Contribution of variables to PCA without CO-04; j-l) Individuals grouped by wine region using confidence ellipses, without CO-04; m-o) Individuals grouped by state using confidence ellipses, without CO-04 (NZ: New Zealand; SA: South Australia; TAS: Tasmania, VIC: Victoria); p-r) Individuals grouped by vintage using confidence ellipses, without CO-04

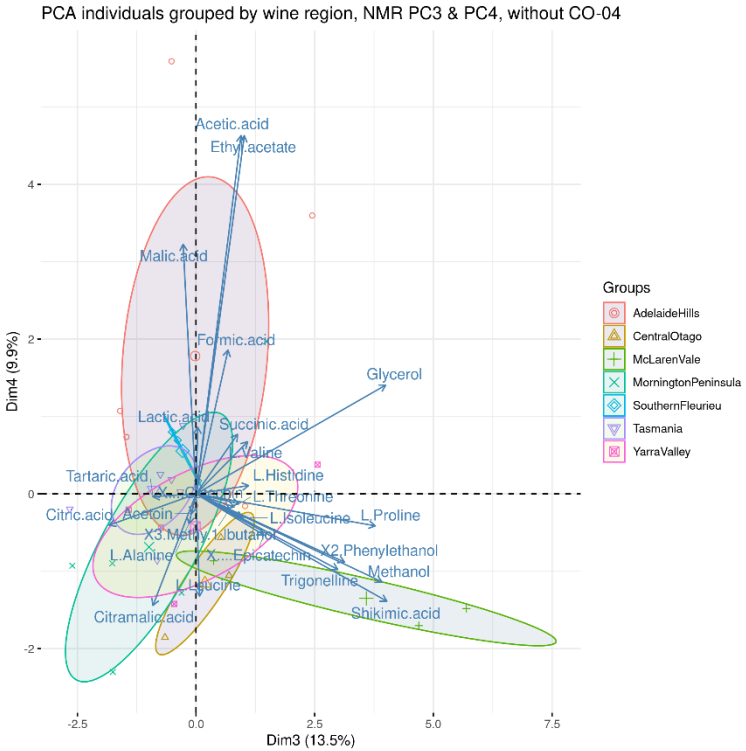


Figure 4.6: Metabolites influencing regional groupings of PC3&PC4

4.3.4 MFA and analysis of coinertia

Multifactorial analysis

The MFA, which compares groups of variables, aimed to determine whether the two chemical analyses (ICP-MS and NMR) agreed with each other. MFA compares groups of variables. Two groups of variables are considered to be close to each other, if two individuals that are close to each other based on analysis of one set of variables are also close to each other in an analysis of the second set of variables (Figure 4.7 a-k). With the analyses of ICP-MS and NMR data, several extreme samples were found: wines AH-02, AH-06, YV-04, and CO-04 showed significantly different data sets compared to all other wines (data not shown).

ICP-MS made the highest contribution to PC1, and NMR to PC2 and PC3 (see Figure 4.7 a-c). Although PC1 and PC2 together only accounted for 37.7% of total variability, they together represented the first two main factors of MFA. PC1 summarised the characteristics of South Australian wines, which were typified by high levels of rare earth metals yttrium, holmium, and dysprosium; and alkaline metals sodium and strontium; as well as the metabolite shikimic acid which is an essential precursor for synthesis of aromatic (aryl) metabolites including flavonoids. PC2 summarises the characters of wines that are determined by NMR, particularly the energy metabolites lactate, succinate, and tartrate; the plant growth hormone trigonelline; and the amino acids proline, histidine, and leucine.

Looking at the specific contribution of variables to PC1, it is clear ICP-MS made the greater contribution (approx. 0.57) to distinguishing wines, though NMR was also of interest (Figure 4.7 a). Yttrium and shikimic acid were the two most important variables, but were closely followed by strontium, and holmium, as well as

alanine and leucine. The rare earth elements were well represented and clearly of interest (Figure 4.7 d). Looking at the specific contribution of variables to PC2, however, it is clear than NMR made the greater contribution (approx. 0.75; Figure 4.7 b). Trigolline, lactic acid, tartaric acid, proline, histidine, and leucine were all important (Figure 4.7 e). PC3 had contributions from NMR, but ICP-MS was also clearly of interest (Figure 4.7 c), with metabolites glycerol and 2-phenylethanol, but also the alkaline metal rubidium all clearly of importance (Figure 4.7 f).

The wines were also categorised into groups using confidence ellipses (see Figure 4.7 i-k), similarly to the ICP-MS and NMR data. Figure 4.7 i shows the wines grouped by region. The South Australian wines from the Southern Fleurieu, McLaren Vale, and Adelaide Hills were quite distinct, although there was some overlap. This was not entirely unexpected, as the regions are contiguous. Figure 4.7 j shows the wines grouped by state. Again, South Australian wines proved distinctive, especially due to MV-01, MV-02, and SF-02. Victorian wines were also reasonably distinctive, particularly due to VY-02, MP-03, and MP-05.

Figure 4.7 k shows the wines grouped by vintage, but as for ICP-MS and NMR there were no discernible patterns, and it was clear that neither ICP-MS nor NMR were able to distinguish the vintage of a wine. This was surprising, given that previous studies have managed to distinguish between vintages [1, 3, 13, 19, 24, 80-83]. However, Greenough, Longerich, and Jackson [2] note that, while their study was apparently able to distinguish between vintages, this may have been an artefact, since wines from the same winery inevitably came from the same vintage, and the elements that correlated with the winery also correlated with the vintage. An attempt to distinguish between different vintages of wines from the same winery was not

successful, and consequently it was concluded that they were not able to distinguish between vintages [2].

The authors consider it possible that the inability of the present study to distinguish between vintages is due to the limited sample size of the study, or to the variation in vintage metabolomic signatures over a large area with differing weather systems; however, further study would be required to determine the reasons for this limitation.

Analysis of coinertia

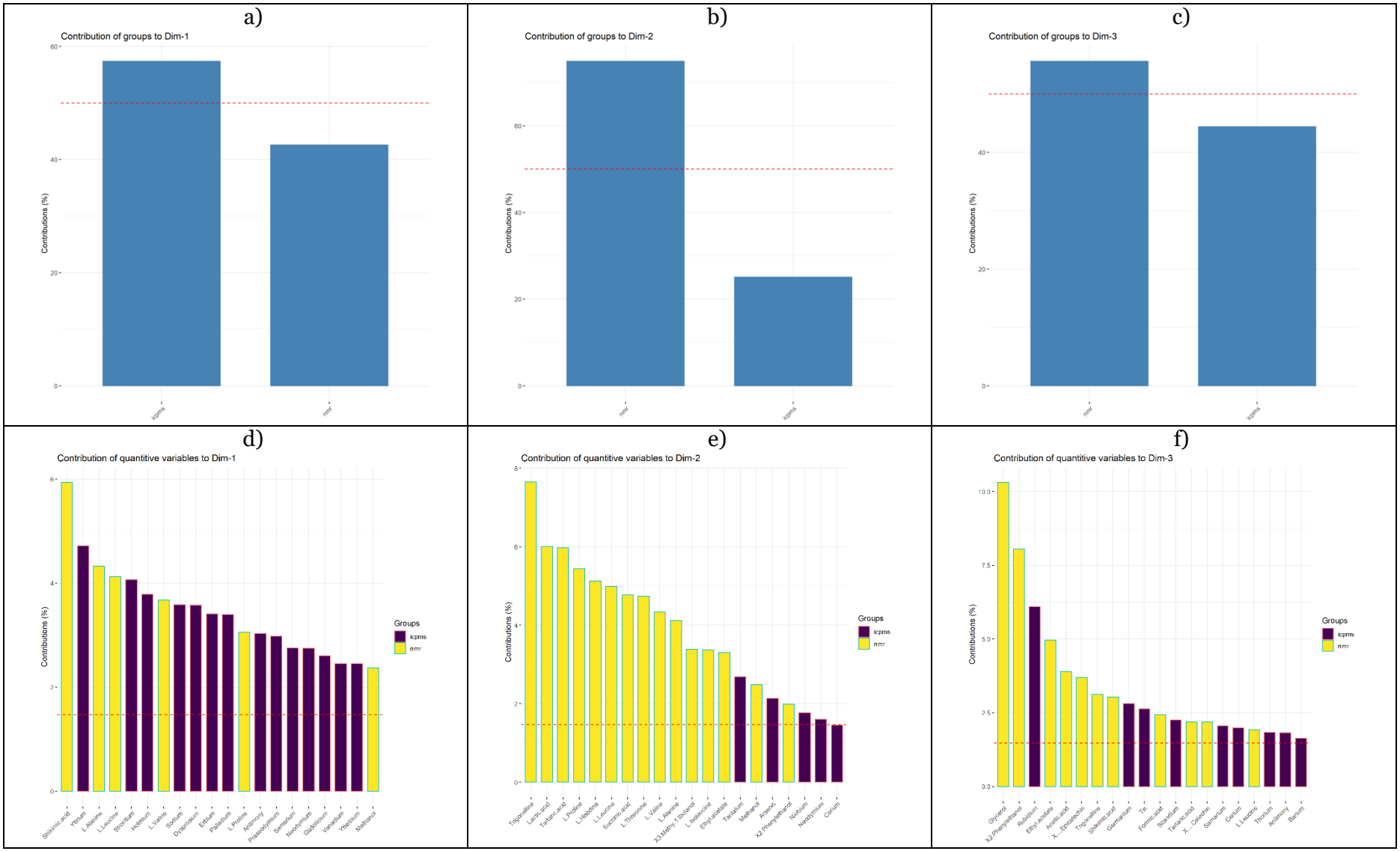
An analysis of coinertia is a two-table ordination method that shows whether the variables from one dataset can be explained by another dataset using a covariance matrix [73, 84]. This is determined from the simulated *p* value, where *p* values below 0.05 (i.e., statistical significance) signify that the variables from one dataset can be used to explain the other.

As with the analyses of ICP-MS and NMR data, several wine samples showing extreme component values were found, and were excluded from the analysis. Three rounds of analysis were undertaken. The first round included all wines (see Figure 4.8 a & d). The second round was run without the extreme wines MV-01, MV-02, and AH-02 (see Figure 4.8 b & e). Finally, the third round was run without the extreme wines MV-01, MV-02, AH-02, AH-06, YV-04, and CO-04 (see Figure 4.8 c & f).

The first analysis provided an *R* value of 0.399, showing how the two new sets of coordinates from the coinertia analysis correlated [73] (see Figure 4.8 a) and a simulated *p* value of 0.015 (i.e. significant). However, this was probably caused by the distorting effects of the outliers, as seen in the row scores (see Figure 4.8 d). The second analysis gave an *R* value of 0.236 (see Figure 4.8 b) and a simulated *p* value

of 0.243 (i.e. not significant). Importantly, two outliers could still clearly be seen in the row scores (see Figure 4.8 e). The third analysis yielded an *R* value of 0.322 (see Figure 4.8 c) and a simulated *p* value of 0.159, also not significant. The row scores finally show any potential patterns or clustering, but none was apparent (see Figure 4.8 f).

These results suggested that the two datasets, NMR and ICP-MS, were not redundant. This was not surprising given that the NMR metabolites reflects winemaking decisions and perhaps vintage effects (though this study was not able to confirm the latter – see the sub-section on Multifactorial analysis, above), while ICP-MS metallic ions derive from vineyard soil and geology [2], as well as winemaking and viticultural techniques (see the sub-section on ICP-MS data, above).


a)
Contribution of groups to Dim-1
Contributions (%)
icpms
nmr
b)
Contribution of groups to Dim-2
Contributions (%)
nmr
icpms
c)
Contribution of groups to Dim-3
Contributions (%)
nmr
icpms
d)
Contribution of quantitive variables to Dim-1
Contributions (%)
Groups
icpms
nmr
Stiiiomic.acid
Yttrium
L.Alanine
L.Leucine
Strontium
Holmium
L.Valine
Sodium
Dysprosium
Erbium
Palladium
L.Proline
Antimony
Praseodymium
Samarium
Neodymium
Gadolinium
Vanadium
Yterbium
Methanol
e)
Contribution of quantitive variables to Dim-2
Contributions (%)
Groups
icpms
nmr
Trigonelline
Lactic.acid
Tartaric.acid
L.Proline
L.Histidine
L.Leucine
Succinic.acid
L.Threonine
L.Valine
L.Alanine
X3.Methyl.1.thujenol
L.Isoleucine
Ethyl.acetate
Tantalum
Methanol
Arsenic
X2.Phenylethanol
Niobium
Neodymium
Cerium
f)
Contribution of quantitive variables to Dim-3
Contributions (%)
Groups
icpms
nmr
Glycerol
X2.Phenylethanol
Rubidium
Ethyl.acetate
Acetic.acid
X...Epicatechin
Trigonelline
Stiiiomic.acid
Germanium
Tin
Formic.acid
Scandium
Tartaric.Acid
X...Catechin
Samarium
Cerium
L.Leucine
Thorium
Antimony
Barium

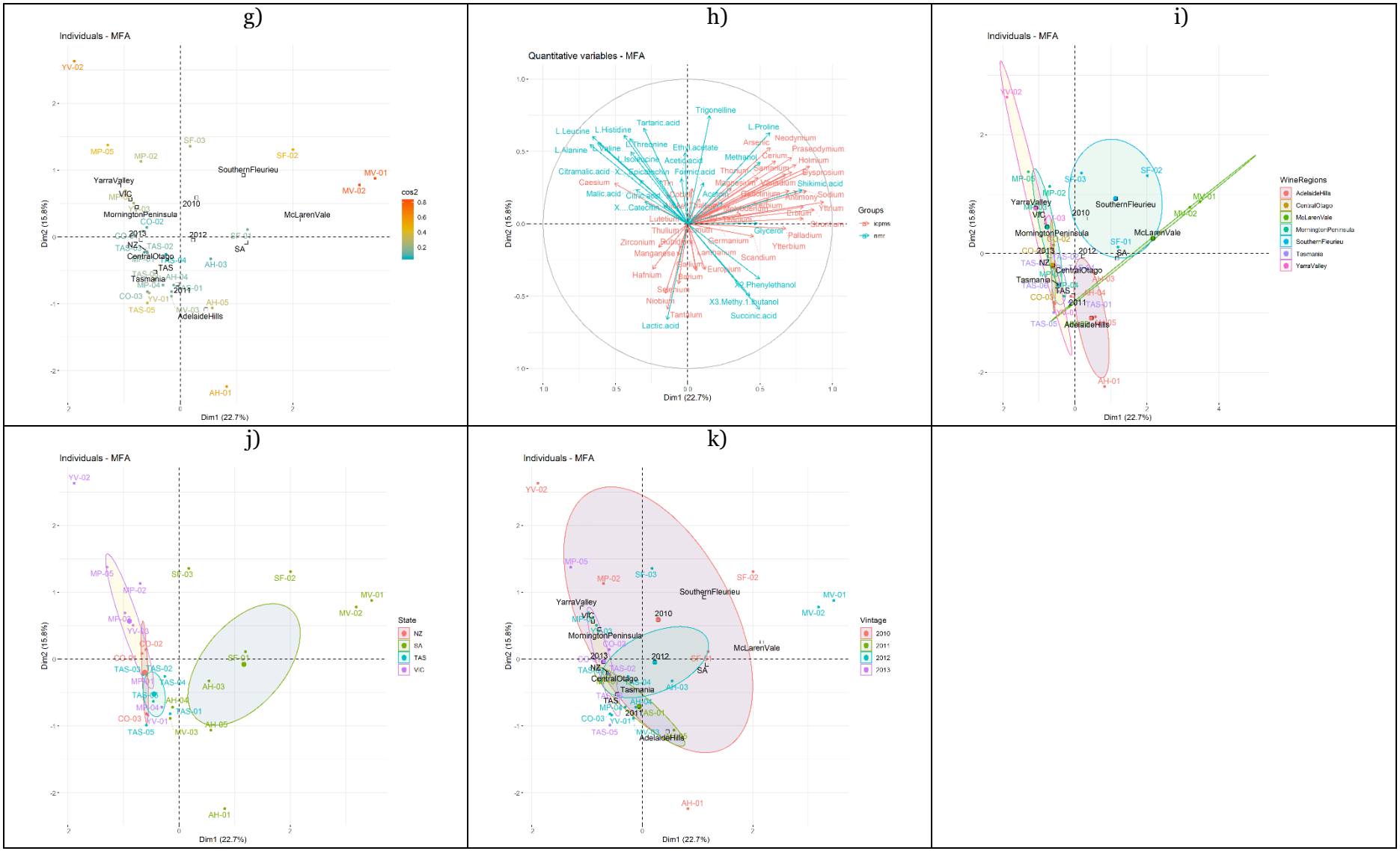


Figure 4.7: MFA analysis

a-c) The contribution of ICP-MS and NMR to the analyses; d-f) Contributions of quantitative variables to PCs 1-3; g) Individual wines in the MFA analysis; h) Influence of the quantitative variable to the analysis; i) Wines grouped by region; j) Wines grouped by state; k) Wines grouped by vintage. Red dashed lines represent 50% of the contribution

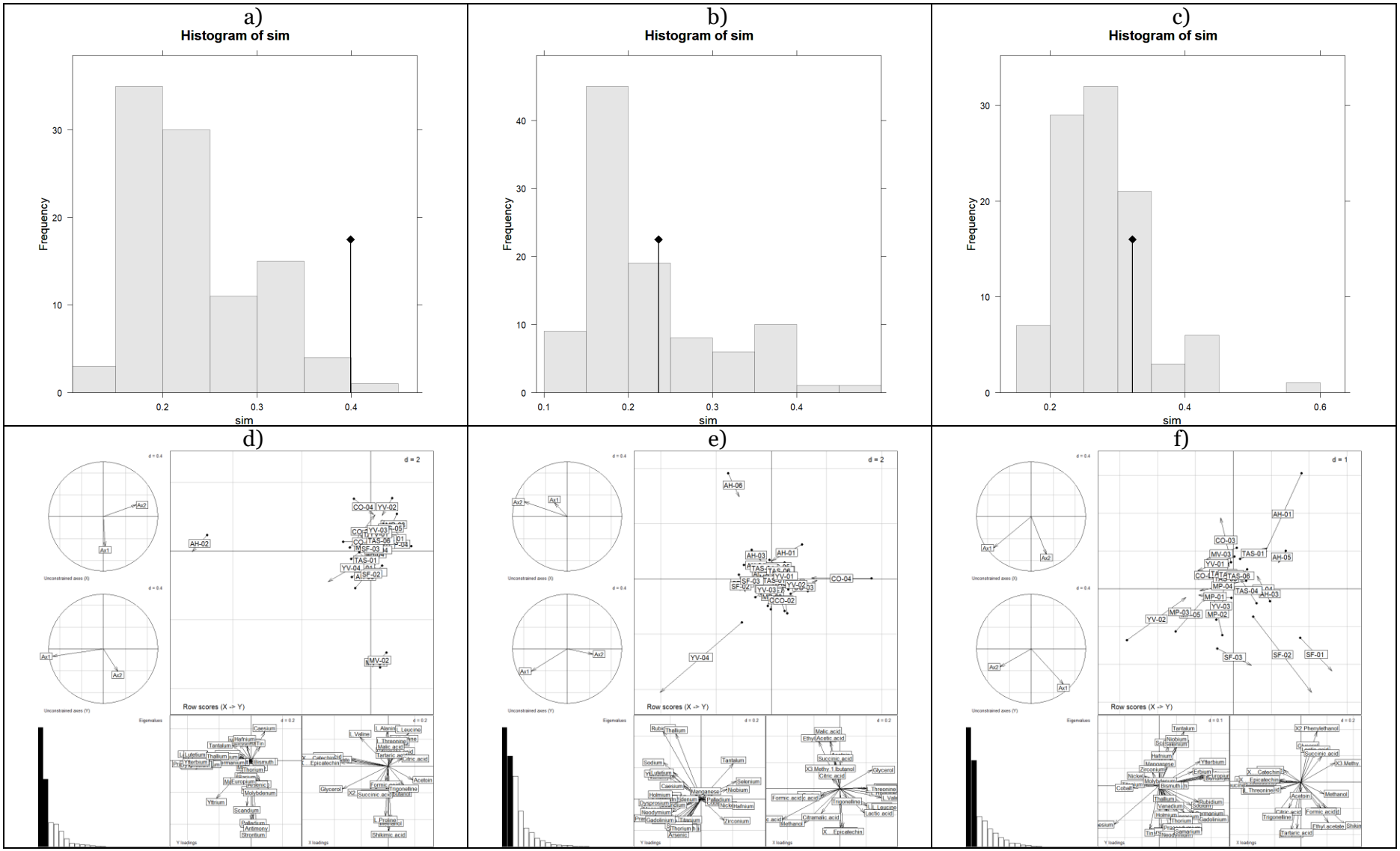


Figure 4.8: Analysis of coinertia

a) Histogram, with extreme samples; b) Histogram, without MV-01, MV-02, and AH-02; c) Histogram, without MV-01, MV-02, AH-02, AH-06, YV-04, and CO-04; d) Analysis of coinertia, with extreme wine samples; e) Analysis of coinertia, without MV-01, MV-02, and AH-02; f) Analysis of coinertia, without MV-01, MV-02, AH-02, AH-06, YV-04, and CO-04. In the histograms, the *R* value is indicated with a straight black line.

4.3.5 Regionality in Australian wine

From this study, it appeared that ICP-MS was a more useful analytical technique than NMR for identifying regions of origin of the bottled Pinot Noir wines from Australia and New Zealand. Combining results of ICP-MS with NMR did not seem to provide any additional discrimination compared with the use of ICP-MS alone.

Given the size, and therefore the heterogeneity, of many of the regions included in the study, it is unsurprising that the regions were not as distinctive as has been shown in other countries. For example, studies in Burgundy have confirmed the distinctiveness of village level appellations [1, 3]. In comparison, Tasmania is an island and an entire state with a landmass of 68,400 km^2. A comparison of more discrete subregions may offer more clear-cut results. It must be noted as well that variable ageing conditions for these bottles may have had an impact on the diversity of metabolites, in particular as seen by NMR.

Equally, the patterns revealed for individual metabolites and minerals may be of interest, and may reward further study. Shikimic acid, a key precursor for the synthesis of aromatic metabolites, may be useful, as may various rare earth elements. In this study, these were both invaluable in separating South Australia from other states.

4.3.6 Suggestions for future work

This research was intended primarily as a pilot study for Australasian wines, using the more modern and highly sensitive analytical technologies of NMR and ICP-MS. Future studies could broaden the study by increasing the number of regions sampled, and the number of samples per region. Ideally, it may be better to restrict the samples to a single vintage, although climatic differences between the widely

separated regions usually vary greatly during any one year. It would also be potentially interesting to determine if vintage differences can be distinguished using a larger number of wine samples, but a smaller number of vintages and a smaller number of regions, preferably regions that are close enough to each other that they experience similar vintage climatic conditions.

Such studies could also be used to test hypotheses regarding the distinctiveness of theoretical Australian wine regions and subregions based on distinctive soil, geology, or climate, alongside sensory studies. It may also assist in determining the distinguishing characteristics of existing regions and subregions. In other countries, similar analyses have also been used to determine wine fraud [13-15, 17, 18, 21-24], and similar work could be undertaken in Australia. Further studies could also determine the utility of NMR in decision-making for winemaking and viticultural techniques during the production of the wine.

Additionally, future work will likely include the establishment of open wine component databases linked to regions and/or specific vineyards; such databases will be invaluable in counteracting wine fraud and are therefore would be of considerable interest to the wine trade.

4.3.7 Concluding thoughts

ICP-MS appears to have promise in determining regionality in Australian and New Zealand wines, perhaps reflecting the extremes in geology often found in these two nations. Although the regional characteristics relating to contributions by *terroir* were frequently overwhelmed by strong local mineral contributions to the wines – possibly resulting from varying soil type, previous mining activity, and viticultural methods such as irrigation – these differences show promise in providing distinctive

'fingerprints' for individual wines. However, NMR may be useful in refining metabolite composition during winemaking and viticulture.

4.4 Acknowledgements

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Chapter 5. General Conclusions

5.1 Introduction

Some overall conclusions can be drawn from the preceding studies that may be of use to grape growers and winemakers both in Australia and internationally. This chapter will summarise some of the key findings and make suggestions for further study.

5.2 Key findings

5.2.1 Higher temperatures may decouple the accumulation of sugars and anthocyanins in Pinot Noir grapes, but this influence is less obvious in finished wines

Pinot Noir is strongly impacted by the climate in which it is grown (Cuccia et al. 2014). The grape is known to grow better in moderate temperatures and with low humidity because it has a thin skin that is prone to fungal attack. Pinot Noir does not grow well in hot climates, and for this reason many areas that were previously ideal for Pinot Noir are becoming too warm with increased temperatures associated with climate change.

However, the results presented in Chapter 2 suggest that higher temperatures may have decoupled the accumulation of sugars and anthocyanins, with the heated treatment grapes producing lower anthocyanin levels than the control treatment grapes, for the same level of TSS.

This influence was less obvious in the finished wines than in the berries, and it is quite possible that other factors may be more important for wine colour, such as sunlight/UV exposure (Matus et al. 2009; Ristic et al. 2007; Song et al. 2015), humidity (Rebucci et al. 1997), soil type (van Leeuwen et al. 2004), and winemaking techniques (Gómez-Plaza et al. 2004; Heatherbell et al. 1996; Pérez-Prieto et al. 2003). This suggests that the impact of climate change on Pinot Noir wine colour,

while not negligible, may be less than previously anticipated (Haeger & Storchmann 2006; Nicholas et al. 2011; van Leeuwen et al. 2013). The influence of climate on wine flavour and aroma, while also critical to wine quality, was beyond the scope of this study.

5.2.2 Bodum presses are of interest for studies involving winemaking

Chapter 3 demonstrated that French press coffee pots or 'Bodum' presses are useful for small scale, replicated ferments. They allow for multiple small fermentations can be easily managed, and the plungers are suitable for replicable cap management and pressing. They are also widely available and inexpensive, allowing for easy procurement. They are therefore ideal for small scale viticulture or oenology studies. This finding is in agreement with Dambergs et al. (2012), and their suggestion of creating small scale 'tank farms' with multiple Bodum presses may be useful for any study that requires statistically sound, replicated experimental fermentations.

5.2.3 Position on the slope is not a confounding factor for small scale winemaking studies

The Bodum press trial (Chapter 3) also determined that the position of vines on a hillside is not a confounding factor in small scale winemaking trials. This provides additional confidence in the results of Chapter 2, as it suggests that the use of a hilly site is unlikely to have introduced an unwanted confounding variable to the study. However, this may vary somewhat depending on the precise aspect and gradient of the slope.

5.2.4 Use of stems in fermentations impacts wine colour and phenolics

Use of stems in fermentations of Pinot Noir grapes was found to have a significant impact on wine colour and phenolics (Chapter 3). This study suggests that Pinot Noir wines made with stems ferments will be more 'brick red' in colour, i.e. that they will be measurably more magenta (higher a* values in the CIElab colour system) and yellow (higher b* values), and measurably darker in colour (lower L* values), than those made without stems. This difference in colour may be due to the higher levels of tannins that found in the wines fermented in contact with the stems (Butler 1981; Dambergs et al. 2012; de la Barra et al. 2005; Goode 2012; Sun et al. 1999). This suggests that the use of stems in ferments may be beneficial for Pinot Noir wine colour; the impacts on wine flavour and aroma, while also critical to wine quality, were beyond the scope of this study.

5.2.5 Australian & New Zealand Pinot Noir wines can be distinguished by region using ICP-MS analysis of metal content, particularly rare earth metals.

In Chapter 4 it was shown that bottled commercial Pinot Noir wines from the southern Australian mainland, Tasmania and New Zealand were able to be distinguished by region using ICP-MS analysis of metallic elements (with rare earth elements being particularly useful). NMR was unable to distinguish between vintages, which was unexpected. Note that Australian wine regions are mostly not defined by soil types, and many of the wines included were blends from multiple vineyards with different soil types.

5.3 Conclusions, limitations, and proposals for further study

It is clear that Pinot Noir grapes and wines are strongly influenced by the climate and soil of the region (and vineyard, at a micro scale) where they are grown (Angus et al. 2008; Greenough, Longerich & Jackson 1997; Nicholas et al. 2011; Roullier-Gall, Boutegrabet, et al. 2014; Roullier-Gall, Lucio, et al. 2014). This can decouple sugar and anthocyanin accumulation, and can impact berry and wine colour, phenolics (as shown in Chapter 2), and metabolites (as shown in Chapter 4). However, it is clear that temperature in isolation is less important for wine colour than previously thought (Chapter 2).

The study on temperature was conducted purely in one vineyard in the Adelaide Hills, South Australia. It is considered likely that similar effects would be observed in vineyards in other regions; however, it may be of interest to replicate this study elsewhere. It would also potentially be of interest to conduct similar studies with other cultivars, including white grape cultivars (one such study had already been undertaken in the Barossa Valley, and provided guidance for this study). It would also be important to determine the influence of climate change on wine quality factors such as flavour and aroma, alcohol levels, and pH, as well as on economic factors such as harvest dates and yields (van Leeuwen & Darriet 2016).

More clear-cut results could perhaps be obtained in a flat vineyard (as opposed to the hilly one used for this study). In addition, studies such as Bonada et al. (2015); Cohen et al. (2012); Sadras, Bubner and Morán (2012); Tarara, Ferguson and Spayd (2000) have used active heating methods instead of the passively heated structures used in this study, and it is possible that this may also provide more clear-

cut results – though it is worth noting that this involves additional logistical challenges, as access to electricity in a vineyard can be difficult, and the amount of energy used by the heating system makes it an expensive option. It would also potentially be of interest to trial some more advanced assays, such as a copigmentation assay.

It is also worth noting that the impacts of climate change will not simply be limited to higher temperatures, since the increased energy in the atmosphere will also lead to an increased frequency of extreme climatic events, such as hail and flooding. These have the potential to dramatically influence viticulture (Cohen et al. 2012; van Leeuwen & Darriet 2016). Further study into methods by which these threats can be mitigated would also be of considerable value.

The study looking at winemaking techniques (Chapter 3) has shown that these too can impact wine colour.

This study examined the use of Bodum presses as fermenters, and found that they are useful for small scale, replicated ferments. The study also determined that the location of the vines on a hillside is not necessarily a confounding factor for a study on wine colour, an issue that was relevant for Chapter 2.

This study also looked at the use of stems during the ferment: a popular, but controversial, method, but did not examine the impact of stems on flavour or aroma. Further investigation would be important given the importance of these factors for wine quality. Equally, a study that examined the impact of stem addition on wine colour, flavour, and aroma during cellaring would also potentially be of interest.

Other winemaking decisions that can influence wine colour may also be of interest for future research: e.g., barrel use (e.g., unoaked *vs* oaked, old oak vs. new

oak, fine grained vs. broad grained oak, barrel size), maceration (time on skins, pump-over vs plunging, maceration techniques such as microwave maceration), or sulfite usage (higher sulfur dioxide levels cause short-term colour loss/bleaching).

The use of ICP-MS to determine regionality in Australian wines was also demonstrated (Chapter 4), and further studies investigating different regions and cultivars may be of interest. The rare earth elements were particularly of interest for distinguishing between regions, and this may also be of interest for future studies (Carvalho et al. 2015; Geoscience Australia 2015; Hoatson, Jaireth & Miezitis 2011; Jakubowski et al. 1999). Metabolomics may prove to be a useful addition to existing techniques such as stable isotope NMR for the detection of wine fraud. This method could determine frauds such as wines from less prestigious regions or cultivars being sold as being wines from more prestigious regions or cultivars.

This study did not find that NMR was able to separate wines based on vintage. The literature is divided on this issue (Ali et al. 2011; Anastasiadi et al. 2009; Cuadros-Inostroza et al. 2010; Greenough, Longerich & Jackson 1997; Lee et al. 2009; Ogrinc et al. 2001; Pereira, Giuliano E. et al. 2006; Pereira, Giuliano Elias et al. 2007; Roullier-Gall, Boutegrabet, et al. 2014; Roullier-Gall, Lucio, et al. 2014), and further study could be of interest. If proved possible, this could also be of interest for wine fraud detection, such as when wine from a poor vintage is sold as being from a particularly sought-after vintage.

Appendices

Appendix 1. Analyses from Chapter 2

Appendix 1.1. ANOVA table for colour (mg anthocyanins per g berry weight) versus total soluble solids, for the linear phase only

```
Analysis of Variance Table for Total Anthocyanins
Response: colour
             Df      Sum Sq      Mean         F        Pr(>F)
                                 Sq       value
brix:tre      2      19.3454     9.6727     236.78     <2.20×  ***
atment                                                 10^-16
Residual     45       1.8383     0.0409
s
---
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Appendix 1.2. ANOVA for wine, CIELab values (3 significant figures)

```
Analysis of Variance Table for L

Response: L

                   Df     Sum Sq   Mean Sq  F value  Pr(>F)
Treatment          1      15.2     15.2     3.61     6.34×10^-2    .
Level              2      79.2     39.6     9.40     3.57×10^-4    ***
Treatment:Level    2      290      145      34.5     5.19×10^-10   ***
Residuals          48     202      4.21
---
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
Analysis of Variance Table for a

Response: a

                   Df      Sum Sq   Mean Sq  F value  Pr(>F)
Treatment          1       117      117      15.4     2.74×10^-4    ***
Level              2       34.8     17.4     2.30     1.11×10^-1
Treatment:Level    2       376      188      24.9     3.86×10^-8    ***
Residuals          48      363      7.56
---
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
Analysis of Variance Table for b

Response: b

                   Df      Sum Sq   Mean Sq  F value  Pr(>F)
Treatment          1       96.8     96.8     53.9     2.23×10^-9    ***
Level              2       73.1     36.5     20.3     4.02×10^-7    ***
Treatment:Level    2       39.8     19.9     11.1     1.11×10^-4    ***
Residuals          48      86.2     1.80
---
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Appendix 1.3. ANOVA for wine, Somer's analysis results (3 significant figures)

Analysis of Variance Table for Total Anthocyanins

Response: Total.Anthocyanins.mg.L

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Treatment	1	3880	3880	13.6	3.565×10^{-4}	***
Level	2	43500	2170	76.5	$<2.20\times10^{-16}$	***
Treatment:Level	2	8370	4180	14.7	2.418×10^{-06}	***
Residuals	102	29000	284			

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Analysis of Variance Table for Colour density

Response: Colour.density.au

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Treatment	1	1.41	1.41	14.7	2.18×10^{-4}	***
Level	2	12.8	6.39	66.7	$<2.20\times10^{-16}$	***
Treatment:Level	2	18.5	9.25	96.5	$<2.2-\times10^{-16}$	***
Residuals	102	9.78	0.0959			

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Analysis of Variance Table for Total phenolics

Response: Total.phenolics.au

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Treatment	1	536	536	251	$<2.20\times10^{-16}$	***
Level	2	233	116	54.5	$<2.20\times10^{-16}$	***
Treatment:Level	2	357	179	83.5	$<2.20\times10^{-16}$	***
Residuals	102	218	2.14			

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Appendix 2. Tables from Chapter 3

Appendix 2.1. Analysis of Variance table for Bodum coffee press trial ferment CIELab data, using GNU R.

```
Analysis of Variance Table for CIELab L*

Response: SlopeCIELAB$L
                          Df     Sum Sq  Mean     F        Pr(>F)
                                         Sq       value
SlopeCIELAB$Wine          2      33.53   16.77    1.143    0.3797
Residuals                 6      88.03   14.67
```

```
Analysis of Variance Table for CIELab a*

Response: SlopeCIELAB$a
                      Df    Sum Sq  Mean    F       Pr(>F)
                                    Sq      value
SlopeCIELAB$Wine      2     47.51   23.76   1.159   0.3754
Residuals             6     123.0   20.50
```

```
Analysis of Variance Table for CIELab b*

Response: SlopeCIELAB$b
                  Df    Sum Sq  Mean Sq  F value  Pr(>F)
SlopeCIELAB$Wine  2     3.688   1.844    1.413    0.3142
Residuals         6     7.831   1.305
---
Signif. codes: ***<0.001; **<0.01; *<0.05; '.'<0.1
```

Appendix 2.2. ANOVA results for HPLC analysis of malvidin concentrations in wine ferments versus slope position.

```
Analysis of Variance Table

Response: SlopeHPLC$MalvidinConc
                Df      Sum Sq   Mean Sq  F value  Pr(>F)
SlopeHPLC$Wine   2      8377     4188     6.000    0.01217   *
Residuals       15      10470    698.0
---
Signif. codes: ***<0.001; **<0.01; *<0.05; '.'<0.1
```

Appendix 2.3. ANOVA tables for CIELab parameters of stemmed vs destemmed wines.

Analysis of Variance Table for L*

Response: StemsCIELAB$L

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Wine	1	11.93	11.93	10.96	0.00441	**
Residuals	16	17.41	1.088			

Analysis of Variance Table for a*

Response: StemsCIELAB$a

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Wine	1	6.207	6.207	6.912	0.0182	*
Residuals	16	14.37	0.898			

Analysis of Variance Table for b*

Response: StemsCIELAB$b

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Wine	1	8.019	8.019	91.39	5.12×10^{-8}	***
Residuals	16	1.404	0.0877			

Signif. codes: ***<0.001; **<0.01; *<0.05; '.'<0.1

Appendix 2.4. ANOVA tables for Somers analysis stemmed vs destemmed wines.

Analysis of Variance Table for Total Anthocyanins

Response: Total.Anthocyanins.mg.L

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Wine	1	95	618.0	27.74	7.75×10^{-6}	***
Residuals	34	757.5	22.28			

Analysis of Variance Table for Colour Density

Response: Colour.density.au

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Wine	1	1.20	1.209	23.90	2.43×10^{-5}	***
Residuals	34	1.71	0.0504			

Analysis of Variance Table for Total Phenolics

Response: Total.phenolics.au

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Wine	1	15304	1530	443	$<2.2\times10^{-16}$	***
Residuals	34	118	3.46			

Signif. codes: ***<0.001; **<0.01; *<0.05; '.'<0.1

Appendix 3. Supplementary materials from Chapter 4

Appendix 3.1. Lab analyses

Note that CIELab measurements were undertaken using a 1mm path cuvette.

Table A1: Lab analyses of wine samples

Wine Code	pH	Free Sulfites (mg L^{-1})	Bound Sulfites (mg L^{-1})	Total Sulfites (mg L^{-1})	Antho-cyanins (mg L^{-1})	Colour density (abs units)	Total phenolics (abs units)	L*	a*	b*
AH-01	3.66	6.4	68.0	74.4	46.2	4.4	25.8	91.7	6.9	5.7
AH-02	3.59	12.0	40.0	52.0	72.3	6.7	53.4	87.1	11.5	7.4
AH-03	3.46	40.8	48.8	89.6	76.4	6.1	29.0	91.6	7.4	5.9
AH-04	3.47	20.8	89.6	110.4	68.1	6.1	34.5	89.6	8.9	6.5
AH-05	3.41	23.2	88.8	112.0	43.9	4.5	45.7	90.5	7.8	6.6
AH-06	3.29	8.0	73.6	81.6	98.0	8.2	30.5	81.0	19.6	3.2
CO-01	3.54	18.1	52.8	70.9	145.2	10.4	39.6	88.2	11.8	3.1
CO-02	3.58	16.0	42.4	58.4	152.1	10.8	43.4	86.5	13.6	2.5
CO-03	3.53	13.6	36.0	49.6	97.9	8.0	39.4	86.8	12.6	4.6

CO-04	3.67	20.0	67.2	87.2	78.7	6.4	30.8	91.5	7.3	6.7
MP-01	3.54	8.8	76.8	85.6	51.8	3.9	32.5	93.9	6.0	5.7
MP-02	3.41	16.0	63.2	79.2	75.7	6.0	45.6	88.1	11.5	8.8
MP-03	3.40	36.8	71.2	108.0	101.6	7.1	38.4	87.6	12.3	5.8
MP-04	3.43	21.6	104.0	125.6	162.4	12.2	42.8	91.4	7.9	5.5
MP-05	3.46	20.8	68.0	88.8	162.4	12.2	42.8	82.0	17.6	5.1
MV-01	3.58	12.0	66.4	78.4	167.7	13.1	52.3	77.2	21.6	8.8
MV-02	3.58	13.6	65.6	79.2	164.4	13.3	49.8	77.4	21.6	8.6
MV-03	3.79	21.6	69.6	91.2	171.8	12.9	42.6	83.7	14.7	5.2
SF-01	3.68	22.4	94.4	116.8	60.3	5.4	21.6	91.1	10.0	6.7
SF-02	3.41	8.0	42.4	50.4	85.5	7.9	48.1	81.8	16.6	6.7
SF-03	3.46	15.2	59.2	74.4	86.9	7.1	40.2	89.4	9.5	6.2
TAS-01	3.59	13.9	50.4	64.3	88.0	6.8	45.2	87.6	12.7	5.5
TAS-02	3.59	19.2	41.6	60.8	101.7	7.7	37.4	89.7	10.6	3.6
TAS-03	3.62	16.0	56.0	72.0	144.1	10.7	31.6	88.7	11.4	4.6
TAS-04	3.43	12.8	36.8	49.6	108.4	8.6	35.6	84.1	16.3	4.9

TAS-05	3.75	16.0	36.0	52.0	107.2	7.9	25.4	90.3	8.6	4.2
TAS-06	3.82	10.4	77.6	88.0	118.9	9.2	31.8	87.0	11.1	4.6
YV-01	3.60	13.6	68.8	82.4	104.4	7.1	43.9	89.5	10.1	6.7
YV-02	3.52	8.0	47.2	55.2	55.7	4.8	38.3	90.5	8.5	6.5
YV-03	3.41	12.8	56.0	68.8	62.5	5.6	28.2	91.6	8.4	5.1
YV-04	3.39	24.0	57.6	81.6	202.7	12.4	38.3	88.5	13.3	3.7

Appendix 3.2. NMR data – cluster analysis

Seven clusters could be determined (Figure 1 b), though these did not correspond to region, state, or vintage. The same clusters are shown in both the dendrogram and the PCA graph, and are described in Table A2

Table A2: Clusters determined using cluster analysis, NMR data without CO-04

Cluster	Composition	High values	Low values
1	AH-01 AH-05	3-Methyl-1-butanol Succinic acid 2-Phenylethanol	Proline
2	MV-01 MV-02	Shikimic acid Proline Methanol Trigonelline 2-Phenylethanol Glycerol	--

3	AH-06	Malic acid Ethyl acetate Acetic acid	--
4	CO-01 MV-03 CO-02 CO-03 & others	--	Ethyl acetate Methanol Threonine Trigonelline Glycerol
5	AH-02	Lactic acid Catechin Epicatechin Glycerol Valine Acetic acid Ethyl acetate	--

6	MP-02 MP-03 MP-05 YV-03 YV-04 SF-03	Citramalic acid Tartaric acid Alanine Leucine Threonine	--
7	YV-02	Isoleucine Histidine Leucine Threonine Trigonelline Valine	--

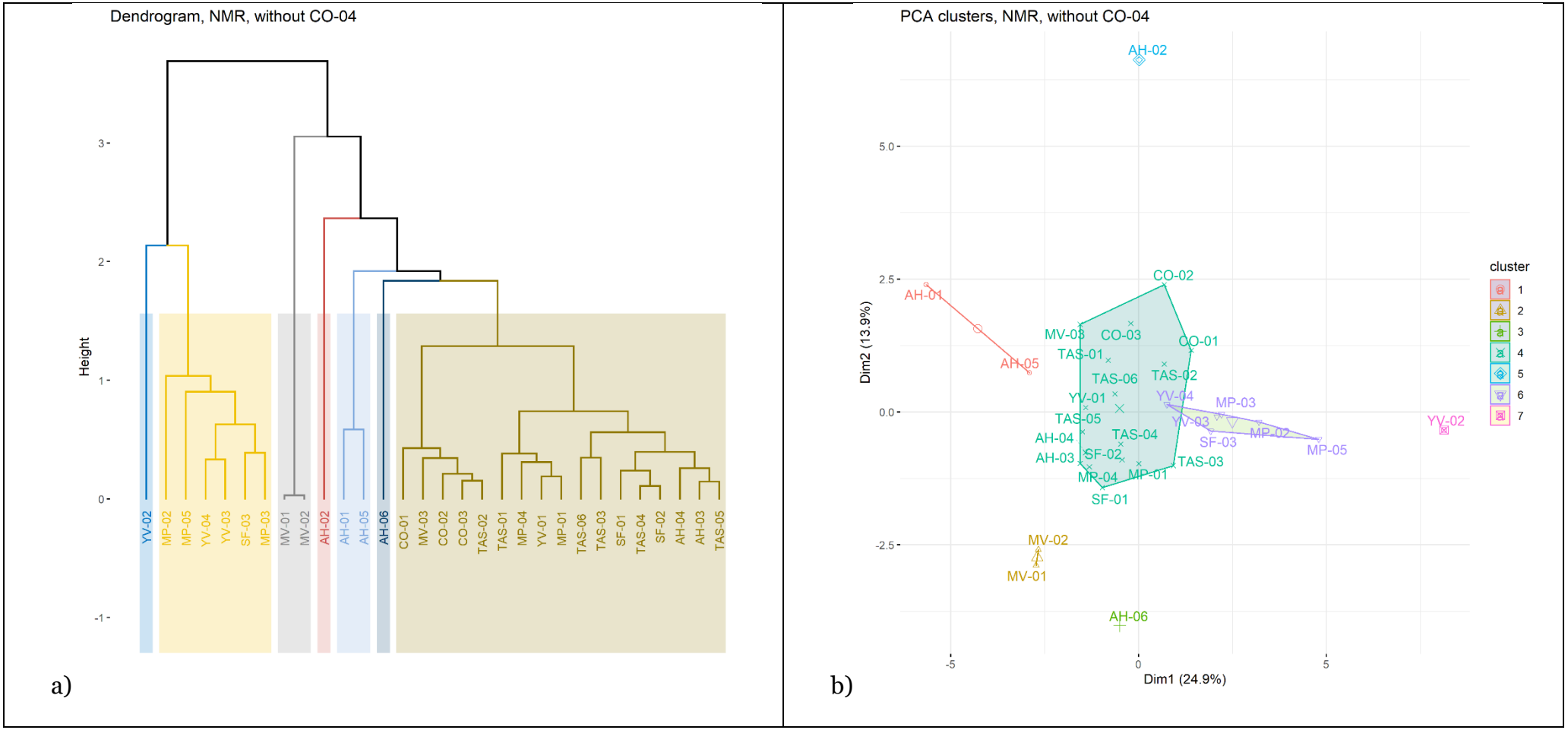

Figure A1: Clusters determined using cluster analysis

a) Dendrogram; b) clusters shown in PC1 & PC2 space

Appendix 3.3. Factors removed from analysis

The following metabolites were removed from the analysis:

ICP-MS:

- Lead Pb (invariate)
- Rhenium Re (invariate)
- Platinium Pt (invariate)
- Tellurium Te (invariate)
- Rhodium Rh (invariate)

NMR:

- Ethanol as % vol (only calculated for one sample, given as g L^{-1} for all samples)
- Acetaldehyde free (invariate)
- 1,3-propandiol (invariate)
- L-aspartic acid (invariate)
- L-glutamine (invariate)
- L-glutamic acid (invariate)
- L-phenylalanine (invariate)
- L-tryptophan (invariate)
- Benzoic acid (invariate)
- Salicylic acid (invariate)
- Sorbic acid (invariate)
- Pyruvic acid bound to SO2 (invariate)

- Ascorbic acid (invariate)
- Ethylvanilin (invariate)
- Vanilin (invariate)
- Trans-cinnamic acid (invariate)
- Acetone (invariate)
- Furfal (invariate)
- Hydromethyfurfal (invariate)
- Sucrose (invariate)
- Melibiose (invariate)
- Raffinose (invariate)
- Rhamnose (invariate)
- L-tyronine (only one above detection levels)

References

- *Note: Chapter 4 is formatted as a paper, and hence its references are separate and not included here. Please see the end of Chapter 4 for a list of references used in that chapter.*

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