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Master thesis

Effects of must turbidity on fermentative aroma development in Sauvignon blanc

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ISA, Lisbon, Portugal and Plant and Food Research, Blenheim, New Zealand are responsible solely for the supervision of completion of the work, not for the contents.

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Heidelberg, Lukas Herrmann

Dedicated to my parents Elisabeth Märkle-Herrmann and Dieter Herrmann.

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3MH	**3-Mercaptohexan-1-ol**
3MHA	**3-Mercaptohexyl acetate**
4MMP	**4-Mercapto-4-methylpentan-2-one**
C	**Carbon**
°C	**Degree Celsius**
CoA	**Coenzyme A**
SBMP	**3-sec-butyl-2-methoxypyrazine**
CO_2	**Carbon dioxide**
DAP	**Diammonium Phosphate**
FTIR	**Fourier transform infrared**
FS	**Free Sulphur**
g	**Gram**
GAE	**Gallic acid equivalents**
GCL	**Gifford's Creek Lane**
h	**Hour**
hl	**Hectoliter**
HPLC	**High-performance liquid chromatography**
kg	**Kilogram**
H_2S	**Hydrogen sulphide**
H_2O_2	**Hydrogen Peroxide**
H_2SO_4	**Sulfuric acid**
IBMP	**3-isobutyl-2- methoxypyrazine**
IPMP	**3-isopropyl-2-methoxypyrazine**
l	**Litre**
mg	**Miligram**
min	**Minutes**
ml	**Mililitre**
mm	**Milimetre**
MP	**Methoxypyrazine**
N	**Nitrogen**
NAD(P)H	**Nicotinamide adenine dinucleotide phosphate**
NH_4	**Ammonium**

NCR	**Nitrogen Catabolite Repression**
ng	**Nanogram**
nm	**Nanometre**
NOPA	**Nitrogen by o-phthaldialdehyde assay**
NTU	**Nephelometric Turbidity Unit**
NZD	**New Zealand Dollar**
OB	**Omaka Block**
PFR	**Plant & Food Research**
ppm	**Parts per million**
PUFA	**Poly-unsaturated fatty acid**
rpm	**Rounds per minute**
S	**Sulphur**
SF	**Superfood**
SFA	**Saturated fatty acids**
SO_2	**Sulphur dioxide**
t	**Ton**
TA	**Titratable acidity**
TCA	**Tricarboxylic acid cycle**
TB	**Tuamarina Block**
UFA	**Unsaturated fatty acid**
V	**Volume**
wt.%	**Weight percent**
YAN	**Yeast assimilable nitrogen**
µg	**Mikrogram**
µm	**Mikrometer**

2.0 Introduction:

In many ways New Zealand has become an ideal for a New World wine country. With the first commercial Sauvignon blanc only released in 1974, Sauvignon blanc fought its way to become not only the flagship of New Zealand`s wine industry, holding around 66 % of the country's total grape production at the 2015 harvest, but also made its way to be recognized for its outstanding quality all over the world. For some critics, New Zealand Sauvignon blanc is arguably the best in the world and others called the Marlborough wine region the best place to grow Sauvignon blanc in New Zealand. Even though testimonies like that may be subjective as taste lies on the tongue of the beholder, New Zealand Sauvignon blanc clearly came a way plastered with success leading to an export value of $1.424 billion NZD in 2015. Key to this success is that the industry found a way to distance itself from its French archetype, world famous Sauvignon blanc originated from the Loire valley, and reinvented itself, introducing a unique winestyle that does not need to shy any comparison. In the heart of this winestyle aroma compounds of the thiol group play an important role. Derived during yeast activity during fermentation from non-odorous precursors found in grapejuice, the two most important thiols that have been related with Marlborough Sauvignon blanc are 3MH and 3MHA. These deliver exotic nuances reminiscent of grapefruit, passionfruit, gooseberry, guava and boxtree. Due to the high oxidability of thiols, several strategies have been adapted by the industry to preserve these key compounds and their non-odorous precursors during the processing of grapes, which includes harvesting, transportation, processing and storage. Although a lot of research has been carried out to further understand the relations between precursors, thiols and their preserving, and to be able to continuously contrast New Zealand Sauvignon blanc from competitors, many questions still remain unclear at the present day. One of them is the effect of juice turbidity on aroma development during fermentation. Research has revealed a broad idea on the positive and negative effects, caused by micronutrients and physical parameters of grape solids derived during process steps leading to juice extraction from the berries on fermentation kinetics and general aroma development. To my best knowledge no research has been published to the present day that focuses on the effect of juice turbidity on the thiol development and expression in Sauvignon blanc ferments. This masterthesis in hand, with the title "Effects of must turbidity on fermentative aroma development in Sauvignon blanc" is supposed to give an overview over the state of research on Sauvignon blanc with a further focus on the question about possible effects and practical applications of juice turbidity. Finally, this work tries to improve the small scale winemaking protocol of Plant and Food Research, Blenheim, where this research has been conducted.

3.0 Bibliographic review:

In 2015 the productive viticultural area of New Zealand was 35000 ha. Although harvest was rather small compared to the 2014 vintage, that led to a total yield of 326,000 tons, export rates were growing during the last years, finally reaching 209 million litres and a record of $1.42 billion in sales. Wine thereby became New Zealand's 6th biggest export good. The most rewarded variety of the country is Sauvignon blanc, reaching a total amount of 66 % of all grapes produced in 2015. 80 % of Sauvignon blanc vines are planted in The Marlborough region, which is known for its big amount of sunshine and cool nights, which preserve acidity and flavours (New Zealand winegrowers, 2015). Typical vegetal and bell pepper flavours which add up to tropical nuances like passion fruit and grapefruit characterize the wines from New Zealand and separate them from wines coming from other origins (Lacey et al., 1991)

The aroma of Sauvignon blanc wines can be generally separated into two groups. One of them, the methoxypyrazines (further referred to as pyrazines) are responsible for the so called "green" aromas. They are generally described as grassy, herbaceous, asparagus, green pepper, capsicum or tomato leaf (Allen et al.,1991; Marais, 1994). Thiols on the other hand bring "yellow", tropical aromas of gooseberry, grapefruit and passionfruit. Composition of these aromas and especially the impression of the final wines are highly connected to the country they come from and even to regions inside the country. Marlborough Sauvignon blanc with its aromas of sweet, sweaty, passion fruit and capsicum characteristics stands out, when compared to other regions in New Zealand. Hawkes bay wines are known for flinty characteristics and Wairapara, known for cat urine and boxwood characteristics (Lund et al., 2005). Lund et al., (2009) showed that Marlborough Sauvignon blanc shows the highest analytical rates of MP and thiols (3MH and 3MHA), when compared to wines, coming from France, South Africa and Australia. Australia Sauvignon blanc are known to have apple, and lolly characteristics while South African and French wines show general notes of minerals, flintstone and bourbon.

Research also showed that the Marlborough style is preferred by New Zealand consumers and is a recognized style all over the world. Although other compounds add to the flavour profile like esters, C6 compounds and fusel alcohols, not all contributors to Sauvignon aroma are known now (Aznar et al., 2003). As consumers, especially more knowledgeable tend to prefer wines coming from specific regional origins, it is very important to increase knowledge about regional differences and find ways to ensure the presence of these parameters for the future. Investment in research programs by the industry, to improve and ensure product quality are known to have a positive impact on price points accepted by customers (Schamel et al., 2006).

3.1 Pyrazines:

Pyrazines are nitroginated ring structures produced as a secondary product of amino acid catabolism in grapes and therefore directly derived (Allen et al., 1991). They are found in stems, grapeskin and seeds, of which the stems contain the highest amount (Roujou de Boubee et al., 2002). Taking a closer look on the grape parts used during white wine vinification, they are located to 67 % in the grapeskins (Ribereau-Gayon et al., 2006). The three MP most common in wine are; 3-isopropyl-2-methoxypyrazine, IPMP, 3-isobutyl-2-Methoxypyrazine, IBMP, and 3-sec-butyl-2-methoxypyrazine, SBMP (Murray & Withfield, 1975).

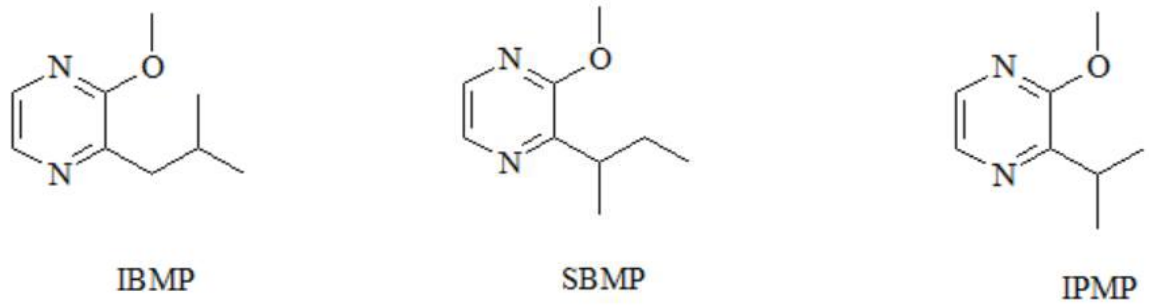

Figure 1: Structural formulas of the three most common Methoxypyrazines.

The main compound contributing to the aroma profile of Sauvignon blanc wines is 2-Methoxy-3-(2-methylpropyl) pyrazine also known as 2-methoxy-3-isobutylpyrazine or 2-isobutyl-3-methoxypyrazine, IBMP (Ribereau-Gayon et al., 2006). It was first detected in Sauvignon blanc wines in 1982 (Augusty et al., 1982). IBMP is detectable at concentrations as low as 2 ng/l but in wines its concentration varies between 12 ng/l and 26 ng/l (Allen et al., 1991). Research of Lacey et al., (1991) detected ranges between 0.6 ng/l and 38 ng/l in wines coming from different origin. IBMP is recognized to bring a greenish, bell pepper aroma, while at lower concentrations, it may only be recognized as a more general vegetal aroma or flavour. Other MP reach much lower values in the final wines. IPMP was reported to be found up to 4.5 ng/l in wines derived from Cabernet Sauvignon and SBMP up to 11.2 ng/l (Sala et al., 2005). It was also suggested, that at higher concentrations, MP may mask other fruity aroma compounds (Chapman et al., 2004).

IBMP was shown to be photodegradable (Heymann et al., 1986) and affected by growing temperature (Lacey et al., 1991). This leads to the conclusion, that viticultural practises affecting canopy architecture and fruit zone microclimate highly affect MP. In fact, higher IBMP levels have to be expected from shaded microclimates. Also higher IBMP concentrations have to be expected from grapes coming from cooler growing regions or years with lower average temperature (Lacey et al., 1991)

MP are known to highly decline with ripening. 96 % loss of the initial IBMP concentration was detected at harvest at maturity stage (Lacey et al., 1991). Similar results were observed by Sala et al., (2005) in Cabernet Sauvignon wines where IBMP concentrations decreased up to 90 % from veraison to harvest. This study revealed similar behaviour other MP, showing a decrease of 94 % for IPMP and 43 % of SBMP.

Research on Cabernet Sauvignon grapes showed, that an increase in yield would decrease IBMP concentrations in the pressed juices. Therefore, pyrazine concentrations correlate like sugars and anthocyanins inverse with crop yields. Also the intensity ratings of bell pepper aroma, vegetative aroma, and vegetative flavour by mouth were all positively correlated with the IBMP concentration in wines (Chapman et al., 2004).

Sala et al., (2005) showed that MP are correlated with planting density. Denser planted vineyards showed higher amounts of MP. Further the effect of irrigation was studied and irrigated vineyards showed to have higher MP levels.

IBMP was less affected by the amount of pressure applied during commercial grape pressing but increased by skin contact (Magu et al., 2007). These results disagree with the findings of Roujou de Boubee et al., (2002) who found that press wines had higher MP levels, which is a more obvious result, as MP are located to higher amounts in the skin than in the berries flesh.

Kotseridis et al., (2008) showed in a trial with different turbidity levels between 25 and 1280 NTU, that 3-isopropyl-2-methoxypyrazine (IPMP) levels correlate with the amount of turbidity left in a juice before fermentation. The amount of IPMP was positively correlated with the time, juice was prefermentative allowed to settle. The usage of bentonite further increased the effect of settling. This research showed also, that the amount of (IPMP) increased in all turbidity levels during fermentation, which was suggested to be a result of grape solid degradation during fermentation. Roujou de Boubée et al., (2002) already showed that Sauvignon blanc juice clarified to 200 NTU lost half of its IMBP amount compared to an untreated version.

3.2 Thiols:

Sauvignon blanc belongs to the so called "simple flavoured" varieties. This means that musts arising from this variety show no or barely any traces of the typical aromatic characteristics of the grape (Peynaud E. 1980). Higher odours only develop during fermentation (Tominaga et al., 1998). The aromatic compounds responsible for that are volatile thiols with additional functional groups as ketones, alcohols and esters (Coetzee & du Toit, 2012). Among the many thiols found, three volatiles thiols have been identified to have a major influence in the typical aroma of Sauvignon blanc: 4-Mercapto-4-methylpentan-2-one, 4MMP (Darriet et al., 1995), 3-Mercaptohexan-1-ol, 3MH (Tominaga et al., 1998) and 3-Mercaptohexyl acetate, 3MHA (Tominaga et al., 1996).

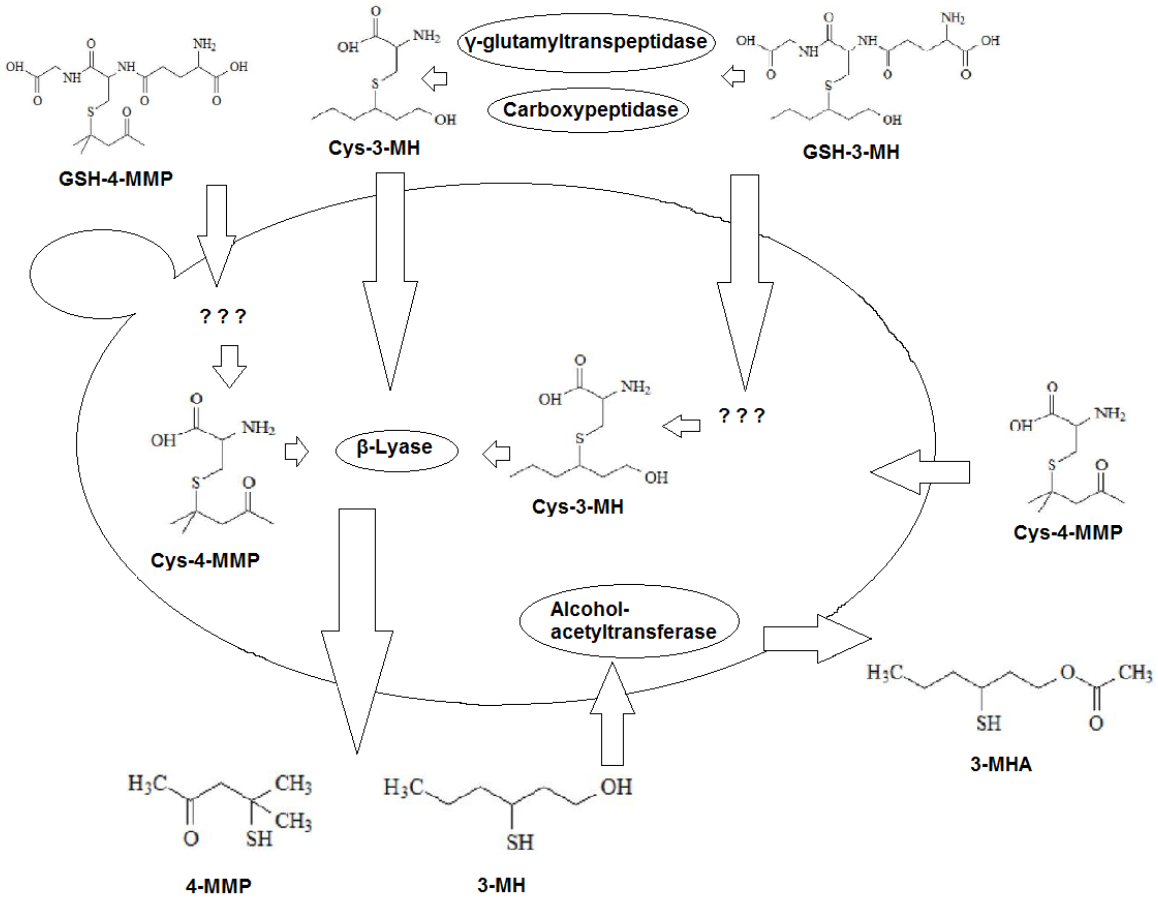

Figure 2: Genesis of the three most common thiols, from their non-odorous precursors by yeast interaction during fermentation.

4MMP gives a smell of box tree, passion fruit, broom, black current, citrus zest and grapefruit, but can also give hints of cat urine, if present in higher amounts (Darriet et al.,1995; Dubordieu et al., 2006). 3MH brings odours of passion fruit, grapefruit, gooseberry and guava (Tominaga et al.,1998) and 3MHA reveals passion fruit, grapefruit, box tree, gooseberry and guava (Tominaga et al., 1996). Thiols have very low sensory perception threshold: 4MMP is the lowest, detectable from 0.8 ng/l. The amount of 3MH needed to be detected is 60 ng/l and finally 4 ng/l are required to make 3MHA findable in wines (Dubourdieu et al., 2006).

Although a lot of research has been carried out during the last years in this field, pathways to free volatile precursors from their non-odorous precursors are not indicated completely yet and the context to final thiol concentration in wine still remains unclear. Therefore, this is still a topic of recent research. Especially the formation of 3MH seems to be quite unpredictable as the precursor formation process is dynamic and affected by multiple factors. Three pathways for the formation of 4MMP and 3MH in *saccharomyces cerevisiae* have been identified so far.

1. The first pathway described, is the direct transfer of the Cysteinlyated precursors: 3-S-cysteinylhexan-1-ol (Cys-3MH) and S-3-(4-mercapto-4-methylpentan-2-one)-cysteine (Cys-4MMP) by beta-lyase (Tominaga et al., 1998).
2. The second pathway describes the presence of S-3-(hexan-1-ol)-L-cysteine in grapes as an intermediate in the breakdown process of S-3-(hexan-1-ol)-glutathione, arising from detoxification processes in vines. Enzymes involved in this process have been suggested to be γ-glutamyltranspeptidase and probably carboxypeptidase first by Gachons et al., (2002). Clear relationship was shown by Roland et al., (2010), using stable isotope dilution assay. This pathway was finally confirmed by recent studies with more sensible methods (Capone et al., 2011).
3. The third pathway is a convertion of (E)-2- hexenal into 3MH and mesityl oxide as a precursor which can be converted to 4MMP. This pathway is proposed to require either the presence of a sulfur donour as H_2S, cystein or glutathion to finally form several possible adducts (Schneider et al., 2006, Roland et al., 2010).

Swiegers et al., (2005) showed that no cysteinlyated precursors exists for 3MHA. This compound is only formed during fermentation by *saccharomyces cerevisiae*. The enzyme shown to be responsible for this transfer is alcohol acetyltransferase, the same enzyme responsible for the formation of the ester ethyl acetate. This study also revealed that the formation of 4MMP does not correlate with the cells ability to form 3MH.

There is a big pool of potential aroma still locked in Sauvignon blanc wines after fermentation, which might be able to be unlocked by winemakers in the future. The amount of 3MH gained from the transformation of Cys3MH represents only 3-12 % of the total amount in wine (Masneuf et al., 2006; Subileau, 2008). Only 10 % of 3MH were shown to arise from green leave volatiles as demonstrated in point 3.) (Schneider et al., 2006).

Recent studies focusing on genes involved in the conversion of cys-4MMP and glut-3MH to their related thiols 4MMP and 3MH showed that the yeast gene IRC7 is essential for this transformation (Thibon et al., 2008). In addition it was shown that the gene OPT1 is required for the uptake of glut-3MH into the yeast cell, although further transporters may be required for this step in some yeast genomes and CIS2/ECM38, encoding γ-glutamyltranspeptidase to further transfer glut-3MH to cys-3MH as part of the detoxification process of electrophilic xenobiotics (Ubiyvovk et al., 2006). The full length IRC7 gene is then not only essential for the release of 3MH but was also found to be the gene responsible for the transformation of cys-4MMP to 4MMP (Santiago & Gardner, 2015). Former research has already revealed the encoding of beta-lyase by IRC7 and have shown a preference of the enzyme towards cys-4MMP to cys-3MH (Roncoroni et al., 2011). Other research also revealed the effect of STR3

to the encoding of beta-lyase, although its effect was found to be rather minor (Harsch & Gardner, 2013). The conversion of cys-3MH to 3MH is only partly affected by the IRC7 gene (Santiago & Gardner, 2015).

The levels of thiols found in bottled Sauvignon blanc wines are strongly influenced by both, viticultural practises as well as winemaking conditions and enological treatments.
It was shown, that precursors greatly increase during ripening, except for the cysteinlyated conjugate of 4MMP, especially in the pre-harvest time (Roland et al., 2010; Capone et al., 2011; Cerreti et al., 2015). Moderate water stress can increase (Cys-3MH) and decrease (Cys-4MMP) concentration in musts, but more severe water stress will lead to a degradation of the final concentration of cysteinlyated precursors (Choné, 2001; Peyrot des Gachons, 2002). To supply the vine with sufficient amount of nitrogen has shown to improve the aromatic potential by increasing cysteinlyated precursor levels (Choné et al., 2006). The foliar application of a combination of sulphur and nitrogen prior to veraison showed to have a positive effect (even a higher effect than a single nitrogen application) on glutathione and volatile thiol expression in wines obtained, without negative impacts on yield and vine vigour. No increase in *botrytis cinerea* was seen in this study (Lacroux et al., 2008).
Besides the many changes occurring by an infection with *botrytis cinerea*, levels of volatile thiols have been shown to increase strongly in wines made from botrytisized grapes both, analytical and sensorial (Sarrazin et al., 2006). Especially S-3-(hexan-1-ol) cysteine (P-3SH) has been shown to be affected strongly by infection with the fungus, due to a stimulation of the grapes metabolic pathway (Thibon et al., 2009).
3MH-S-cys levels were seen to increase when higher pressures were applied during the pressing of grapes. Also skin contact showed an increase of 3MH-S-cys, precursor for varietal aroma (Magu et al.,2007; Roland et al., 2011). These observations are explainable by the fact that more than 50 % of 3MH precursors are found in grapeskins, though 80 % of 4MMP precursors are found in the berry flesh (Peyrot des Gachons, 2002). An extraction of polyphenols though was shown to have an indirect negative impact on thiols. Research by Blanchard et al., (2004) revealed higher oxidation of 3MH in the presence of oxygen and catechin compared to the presence of only oxygen. It was shown that must oxidation affected hydrocinnamic acids which form quinones and usually later react with glutathione via a Michael addition, to GRP (grape reaction product) due to the presence of phenoloxidase activity under presence of oxygen on hydrocinnamic esters, particularly trans-caftaric acid (Singleton et al., 1985; Singleton, 1987). But quinones are also able to directly react with thiols within a Michael addition or form peroxides as a result of multiple reactions (Wildenradt & Singleton, 1974; Cheynier et al., 1986; Nikolantonaki & Waterhouse, 2012). Nikolantonaki & Waterhouse, (2012) showed also that thiols react differently with quinones, 4MMP was shown to be less

affected than 3MH. The thiol-oxidative capacities of peroxides have more recently been confirmed (Blanchard et al., 2004). Must oxidation still seem to be a way to increase G3MH and Cysteinlyated precursors and G4MMP are not decreased, as their sulfhydryl group is involved in a C-S bond which preserves them from oxidation (Roland et al., 2010). These results have to be rated with caution though as they may only match wines with a very low polyphenol fraction. No effect has been seen on different pH and potassium levels in Sauvignon blanc juice (Grose et al., 2015). But a higher oxidation of polyphenols is known to occur at higher pH, which ends in an indirect negative effect on thiols as described above. The use of SO_2 is known to prevent phenol oxidation by inhibition of phenoloxidases and is therefore an important factor of conserving thiol oxidation.

Saccharomyces cerevisiae offers different abilities to free volatile thiols from their non-odorous precursors, especially in their aptitude to release 4MMP from its cysteinlyated precursors. Research by Howell et al., (2004) revealed abilities of certain yeast strains to release 4MMP from its cysteinlyated precursor up to 138 fold in synthetic medium, compared to a control strain. It was shown that the release of 3MH is not affected by the yeast strain. The most efficient commercial wine yeast can only release approximately 5 % from precursors (Murat et al., 2001; Swiegers et al., 2006). In another study, a transfer rate between 0.6 % and 10.2 % with an average of 3,2 % was shown in rose wines coming from AOC Bordeaux (Murat et al., 2001b). Studies have also shown that some non-*saccharomyces* strains may contribute to the final volatile aroma concentrations found in wines, especially to the amount of 3MH released (Zott et al., 2011).

Masneuf-Pomaréde et al., (2006) clearly demonstrated the effect of fermentation temperature on volatile thiol transfer. 4MMP aswell as 3MH were found to be higher when fermented at 20 °C, compared with levels achieved at 13 °C fermentation temperature. In contrast Swiegers et al., (2006) found that a wine fermented at 18 °C had higher amounts of 4MMP than wines fermented at 23 °C and 28 °C. Studies of Howell et al., (2004) on the effect of fermentation temperature on the abilities of different yeast strain to release 4MMP have shown that temperature was an increasing factor for some strains but not for all, leaving the conclusion that other factors need to be considered to qualify the total effect of temperature.

Former research has proven the positive effect of low fermentation temperatures (10 °C) on fruity esters as isoamyl acetate, isobutyl acetate, ethyl butyrate, hexyl acetate compared to the formation of more "heady" esters as ethyl octanoate, 2-phenethyl acetate and ethyl decanoate at higher temperatures during fermentation at 15 - 20 °C (Killian & Ough, 1979). Additionally, the research conducted by Masneuf-Pomaréde et al., (2006) also showed an increase in acetic acid in wines, fermented at higher temperatures. More research has to be carried out to find perfect fermentation temperatures at which wines would profit from both, fruity esters and volatile thiols, leading to a maximisation of total winearoma.

Subileleau et al., (2008) showed that the addition of diammonium phosphate decreases 3MH production. This is suggested to be an effect of the yeast cells nitrogen catabolism repressing GAP1, an identified transporter molecule, responsible to transport Cys-3MH into the cell. This suppression can be extended under nitrogen rich conditions. These findings have also been confirmed more recently by Harsch & Gardner (2013) who found that thiol yields are not only modified by nitrogen containing but also sulphur containing supplements. Among 17 genes involved in sulphur amino metabolism, MET17, CYS4, and CYS3 were standing out, responsible for the multiple step conversion of H_2S to cysteine. Nitrogen wise, addition of ammonia, known to induce nitrogen catabolic repression and urea increased thiol production substantially. These results disagree with the findings of Subileau et al., (2008), who found that only addition of Urea had a positive effect on thiols whereas addition of DAP decreased Thiol expression. Thaillandier et al., (2007) showed different behaviour towards nitrogen, when monitoring four yeast strains. Therefore, effects of nitrogen content and source have to be related to a specific strain. The addition of nitrogen sources has on the other hand shown to have an effect on the final ester profile of wines, as addition of ammonia increases concentrations of acetate esters and ethyl butyrate but not the higher molecular weight fatty acid ethyl esters. Differences depend on the yeast strain added and source of nitrogen used. A general increase on esters by ammonia addition could not have been proven (Miller et al., 2007). Also Pinu et al., (2013) have monitored changes in ester profile due to the addition of nitrogen sources. Further an increase of negative perceived aroma compounds as methional (sweet soup and meat aroma) and 4-ethylguaiacol (glove spicy, smoky aroma) could be monitored.

Recently, the presence of the two precursor Cys-3MH and GSH-3MH was proven in commercial, grape derived tannin (Larcher et al., 2013). Tannins are widely known to winemakers for their abilities to stabilize colour in red wine making and to increase mouthfeel and aroma profile. The positive potential of this thiol source as a contribution to the total precursor pool, leading to higher 3MH and 3MHA values, has been shown in recent research (Larcher et al., 2015). Although more research has to be carried out about the perfect usage of this new precursor source, an alternative, introduced precursor source was revealed within this research.

3.3 Turbidity:

The composition and amount of juice lees, varies with the raw material the juice was obtained from and the process of juice extraction. This refers mainly to total amount and particle size. The lees, obtained after the pressing of grapes consist of earth, skin, stem fragments, cellular

debris, from grapepulp and insoluble residues from vineyard treatment products (Ribereau-Gayon et al., 2006b).

Although the exact chemical composition of grape lees is hardly known, they contain insoluble polysaccharides, few nitrogen compounds, mineral salts and a big amount of lipids, most likely from cellular membranes (Ribereau-Gayon et al., 2006b).

Alexandre et al., (1994) analysed particles obtained by settling of a white must and found they consist of 72 % of total sugars, 8 % of lipids, 5.5 % of minerals, 5.2 % of pectin, and ~2.6 % of nitrogen. Further the author investigates UFAs and detected that linoleic acid is the most abundant UFA in lees, reaching up to levels of 25 %, followed by stearic acid up to 22.2 %, oleic acid and palmitoleic acid to 5.5 %. For SFAs an amount of 25 % of palmitic acid, 13.8 % of stearic acid, and 8.3 % of lauric acid was detected. Tumanov et al., (2015) investigated the lipid composition in a total of 217 Sauvignon blanc juice samples, delivered by wineries all over New Zealand. It was shown, that the total lipid content in grape juices reached up to 2.8 g/l. The free fatty acid content, as directly usable by *saccharomyces cerevisiae* was in a range between 0.56 - 28.45 mg/l. The study revealed also vintage effects on total fatty acid amount and composition of the fatty acid profile. The composition of UFAs and SFAs was previously investigated by Roufet et al., (1987) who found a composition of 72 to 28 % at maturity. During grape maturation, lipids acted quite stable, except for linoleic acid, which proceeds to decline from veraison. Nicolini et al., (2011) suggest a turbidity of 100 NTU to achieve fruity fermentation notes while ensuring a non-sluggish fermentation. Fermenting at slightly higher NTU may lead to a slightly more complex aroma. Ribereau-Gayon et al., (2006b) gave an optimum value between 100 and 250 NTU, Charrier et al., (2013) recommend values between 50 and 150 NTU.

The effects of suspended solids can be separated into two main groups, physical or chemical. The physical component is mainly based on an increase of inner surface. This increase allows CO_2 to be released from the ferment, providing gushing points. At low turbidity though, CO_2 is one of the main inhibitors of yeast cells. Although the actual mechanism of CO_2 on yeast cells could not be proven so far, the most probable explanation is due to an intracellular decrease in pH caused by CO_2 dissolving into the cell membrane, forming carbonic acid. The effect is a decrease in the cytoplasmatic pH, affecting enzymatic activity and protein synthesis and causing irreversible protein denaturation which ultimately leads to the inactivation of the microorganism (Spilimbergo et al., 2005).

Suspended solids are known to have an absorbable effect on various components. Research by Joshi et al., (2013) has revealed a decrease in titratable acidity, related to adsorption by insoluble solids added to an apple wine ferment. Probably this is due to providing crystallisation points for acidic salts. A decrease of K and Na ions can be related too, as residues of K and Na were lower in wines, fermented with solids. Wines with lower K and Na

levels are known to be less likely to tartar instability which can be seen as a positive effect. At the same time suspended solids affected metal ions: a reduction of Cu and Fe content was observed, Zn content increased significantly. Especially degradation of Fe and Cu ions can be seen as positive effects of juice turbidity as those ions are known to have an effect on aroma composition, especially on sulphur containing compounds as thiols and even trace amounts below 1 µg/l were suggested to catalyse thiol oxidation (Blanchard et al., 2004).
Solids in juice are known to contain an amount of polyphenols which are likely to be oxidized by phenoloxidase and to increase colour or may even oxidise thiols by a multiple step reaction via quinones (Joshi et al., 2013).
Joshi et al., (2013) reported results similar to earlier findings which showed a decrease in total ester content in wines fermented from juices with a higher content of solids. These results differ from previous research by Groat & Ough (1987), who found esters and fusel oils to generally increase with higher content of solids fermented.

Research has shown that the prefermentative addition of grape derived solids to grapejuice decreased acetic and pyruvic acid production, reduced the lag phase, and increased the fermentation rate. These effects have been compared to alternative products to increase turbidity as bentonite, diatomaceous earth powder, charcoal, cellulose, gelatine and silica gel, and inert insoluble materials as glass beads, quartz powder, and talc. But none of these alternatives had an effect as strong as grape derived solids. These effects have been related to fatty acid composition of grape solids. In strongly clarified musts acetic acid has been considered to be hydrolised from Acetyl-CoA, unable to be metabolised by yeast (Delfini & Costa., 1993).

Depending on spray timing, active ingredient used and half time of the ingredient, freshly pressed grape juice may contain some pesticide residues. Some early organic fungicides, used to fight downy mildew (*Plasmopara viticola*), Folpet, Captan, Captafol and Dichlofluanid, which are still common to use, have been shown to have antiseptic effects on yeast (Cabras et al., 1987). Fermentation delays have been observed under influence of some pesticides like thiophanate-methyl and fenarimo (Zironi et al., 1991). Molecules nowadays can only be introduced to the market after their inactivity on microflora has been proved. In grapejuice, pesticides normally undergo absorption by yeast, degradation by the acidity of the juice itself or transformation during fermentation. Clarification decreases pesticide residues up to complete elimination (Cabras & Angioni, 2000). Before the introduction of organic pesticides, copper and sulphur were the most abundant ingredients to fight mildew diseases. Copper is naturally found in grapes up to 2 mg/l. Darriet et al., (2001) revealed that the application of copper around veraison into the fruitzone can increase copper concentration in grapes up to 37.6 mg/l. Due to this increase varietal thiols, as found in Sauvignon blanc, Merlot and

Cabernet Sauvignon were decreased strongly in all the three years analysed. 3MH decreased 37 - 94 %, compared with Folpet treated vines, 4-mercapto-4-methylpentan-2- one decreased 25 - 50 %. Finally, this lead to wines lacking in olfactory typicity. It was further suggested that copper is adsorbed by grapesolids and therefore decreased in must after clarification. Depending on the origin of the grapes and the methods applied during their production, residue pesticides may become a stress factor for yeast. Residue copper has a strong effect on thiol yield, if insufficient clarification is carried out.

In research, studying the effect of different juice turbidities between 15 and 350 NTU, it was shown, that an increase in turbidity, decreased fermentation time and residual sugars. The same study could not show differences in ethyl acetate. At the same time a decrease of acetic acid was revealed in ferments of higher turbidity (Nicolini et al., 2011).

Saccharomyces cerevisiae is known to change its metabolism according to changes in environment. Studies showed that yeast also utilises secondary nutrients besides its main metabolites glucose and fructose. Sugar alcohol, carboxylic acids and fatty acids are utilised after fermentation of main sugars which shows that wine is affected by juice composition (Pinu et al., 2014)

3.4 Sterols and fatty acids:

Winemaking techniques as used in countries considered as “new world” wine regions nowadays are known for a highly reductive style of winemaking when it comes to the production of Sauvignon blanc wines. Modern techniques as stainless steel vats, the use of inert gases, sparging, and the use of antioxidatives are used to prevent oxidase activity and the loss of highly reactive thiols, which lead to the appreciated styles of wines coming from “new world” origins. These techniques can lead to juices with an extreme prefermentative prevention of oxygen. Additionally, prefermentative clarification is applied after pressing. Both techniques may cause troubles during the process of winemaking. Already early in the history of wine related research, growth rates of yeast in an oxygen depleted medium were described as very low. Further, the supplementation of sterols was first described to significantly increase yeast growth under such anaerobic conditions (Andreasen & Stier, 1953). More recently the sterol needs of yeast were monitored more precisely, specifying the sterol requirement for optimal yeastgrowth from 2 to 4 mg phytosterols/l (Deytieux et al., 2005). The cell wall of yeast consists mainly of fluidity providing phospholipids (principally phosphatidylcholine and phosphatidylethanolamine) and rigidity providing sterols, principally ergosterol and zymosterol (Walker G.M., 1998). Therefore, lipids and sterols are key factors of the growth, metabolism, and viability of yeastcells during the alcoholic fermentation.

Yeast cells can synthesize their own sterol, ergosterol, in the membrane of the endoplasmatic reticulum by squalene cyclization, from where it is transported through the Golgi bodies to the plasma membrane in aerobic conditions (Zinser et al., 1991). The requirements of oxygen in a lipid depleted must were estimated at about 10 mg/l to maintain a vital yeast population (Sablayrolles & Barre, 1986.) The enzyme responsible for the regulation of sterol synthesis is hydroxymethylglutaryl-CoA reductase. The activity of this enzyme is highly oxygen dependent. Therefore, yeast becomes auxotroph to sterols and long chained fatty acids under anaerobic conditions (Jacquier & Schneiter, 2012). Besides that, UFAs, especially linolenic acid have been shown to accelerate sterol synthesis up to 7 fold. Therefore, it was suggested, that the supplementation of UFAs leads to a completion of the protein synthesizing apparatus during cell growth (Boll et al., 1980).

Only under anaerobic conditions, yeast is able to import exogenous sterols, arising from sources other than fungal (Zavrel et al., 2013). It was shown that the highest UFA uptake rates are observed in the exponential phase of yeast growth, ending in an almost complete consumption (Duan et al., 2015). Luparia et al., (2004) pointed out the importance of solids for nutritional purposes, mainly for their content of grape phytosterols. Casalta et al., (2016) analysed solids from white and red musts and found phytosterol concentrations to range from 3 to 10 mg cholesterol equivalent per gram dry weight. Their composition was described as follows: 89 % β-sitosterol, 6 % campesterol, 3 % stigmasterol, and 3 % stigmastanol.

Betrand and Miele, (1984) have shown that clarification reduces the total fatty acid content in juice to 90 %. Further they suggested that also exogenous sterols are removed within the suspended solids. It was shown that yeast loses its ability to divide when no sterols and UFAs are left in a media to ferment, as these compounds are a non-replishent compound of the cell membrane (Monk, 1997). If yeast is inoculated, 4 to 5 doublings are required to reach the stationary phase but if spontaneous fermentation is allowed, up to 16 doublings are possible. Reserve sterols in the yeast dilute with each doubling, finally leading to cell walls unable to protect its permease proteins, and leading to a rapid acidification of the cytoplasma. Acidification of the cytoplasma finally ends in ethanol induced nutrient leakage and inhibit sugar transportation systems (Monk, 1997; Boivin et al., 1998; Jackson, 2008). In a sterol free medium, in absence of oxygen, yeast cells exhibited stuck fermentation, which is related to a rather low biomass (Luparia et al., 2004). A supplementation of UFAs on the other hand showed to improved yeast growth and fermentation activity (Duan et al., 2015).

Further, the ability of yeast to produce and tolerate increasing ethanol content during fermentation is highly linked to the amount of UFAs and sterols in their plasma membranes (Rose, 1993). *Saccharomyces cerevisiae* was shown to increase UFAs/SFAs and ergosterol

content at the expense of the steryl ester pool in in their cell membranes as a way to adapt to stressful conditions during fermentation (Rupčić J. & Jurešić G.Č., 2010). Finally, Tesnière et al., (2013) showed that lipid limitation affects nitrogen metabolism. A rapid loss of cell viability and higher cell death rates were recorded in a lipid depleted medium, and correlated to higher nitrogen availability. These results implement a possible negative role of nitrogen during fermentation by modulating stress responses. Higher lipid contents were suggested in high nitrogen ferments.

Smith et al., (1996) have shown that ergosterol is not only incorporated by the yeast as part of the cell membrane, but also has effects on gene expression and enzymatic activity. Research by Duan et al., (2015) clearly showed the positive effect of UFAs on most volatile compounds. The amount of higher alcohols (2-phenylethanol, 2-methyl-1-propanol and 3-(methylthio)-1-propanol), medium-chain fatty acids (butanoic acid, hexanoic acid and octanoic acid), acetate esters (isoamyl acetate and 2-phenylethyl acetate) and all ethyl esters was increased after the addition of UFAs to a synthetic medium. As mentioned before, the enzyme responsible for the formation of ethyl esters, alcohol acetyltransferase is also responsible for the formation of 3MHA from 3MH it is expectable that an increase of UFAs could increase the formation of this compound. To my best knowledge, no research about the effect of UFAs on 3MHA formation was done to date.

Pinu et al., (2013) suggested that the initial biosynthesis of 3MH might be influenced by the activity of the TCA cycle and fatty acid metabolism of yeasts as linoleic acid has been shown to correlate with 3MH even if no correlation was found in this study with 3MHA or its acetylation ratio. *Saccharomyces cerevisiae* lacks of ∆12-fatty acid desaturase and ω3-fatty acid desaturase, the enzymes required for the production of polyunsaturated fatty acids as linoleic acid and linolenic acid (Yazawa et al., 2009). Research has revealed the contribution of juice lees to content in linoleic and linolenic acids and related enzymes (Nicolini et al., 2011; Varela et al., 1999). The contribution of PUFAs to cell integrity and a wide stress tolerance is widely known (Kajiwara et al., 1996; Rodriguez-Vargas et al., 2007; Yazawa et al., 2009).

The formation of hexanal and hexenals as (Z)-3-hexenal and (E)-2-hexenal due to the enyzmatic breakdown of linoleic acid was formerly described by Drawert, (1974). The pathway to form n-hexanal from linoleic acid was described by Matsui, (2006) as a result of lipoxigenase (LOX) being able to oxidise the carbon double bonds of linolenic acid, forming linolenic acid 13-hydroperoxide (13HPOT). Further 13-hydroperoxide lyase (13HPL) is able to form (Z)-3-hexenal and 12-oxo-(Z)-9-dodecenoic acid. LOX is known to be able to break doublebonds at position 9, preferring linoleic acid (100 % activity) over linolenic (60.4 % activity) and oleic

acids (46 % activity). It was further shown, that the highest activity of this enzyme occurs between 25 and 30°C and an increased activity was monitored in presence of magnesium ions (Busquets et al., 2004). Drawert (1974), described these enzymatic-oxidative processes occurring after the crushing of fruit, if oxygen had access to the substrate and if enzymes had not been inhibited previously and the formation of these compounds leading to grassy, green impression of freshly crushed unripe grapes and leaves. This effect was also described by Roufet et al., (1987) who described the proceeding decrease of linolenic acid during maturation as the reason, that green, unripe, unpleasant flavours are not to be found in juices pressed from ripe fruit. Harsch et al., (2013) proved that a sulphur donor as H_2S is required to further transfer (E)-2-Hexenal and its alcohol (E)-2-Hexen-1-ol into 3MH and 3MHA. It was also shown that (E)-2-Hexenal and (E)-2-Hexen-1-ol are metabolized in the first 24 hours after yeast inoculation, prior to alcoholic fermentation, probably to decrease its fungicide effect, which was mentioned by Kubo et al., (2003).

At the other hand it was indicated that linoleic and linolenic acid influences ester and medium chain fatty acid excretion negatively when fatty acids in the yeast reached a defined concentration. The same study revealed an increase in fusel alcohols when linoleic and linolenic acid were increased (Rosi and Bertuccioli, 1992).

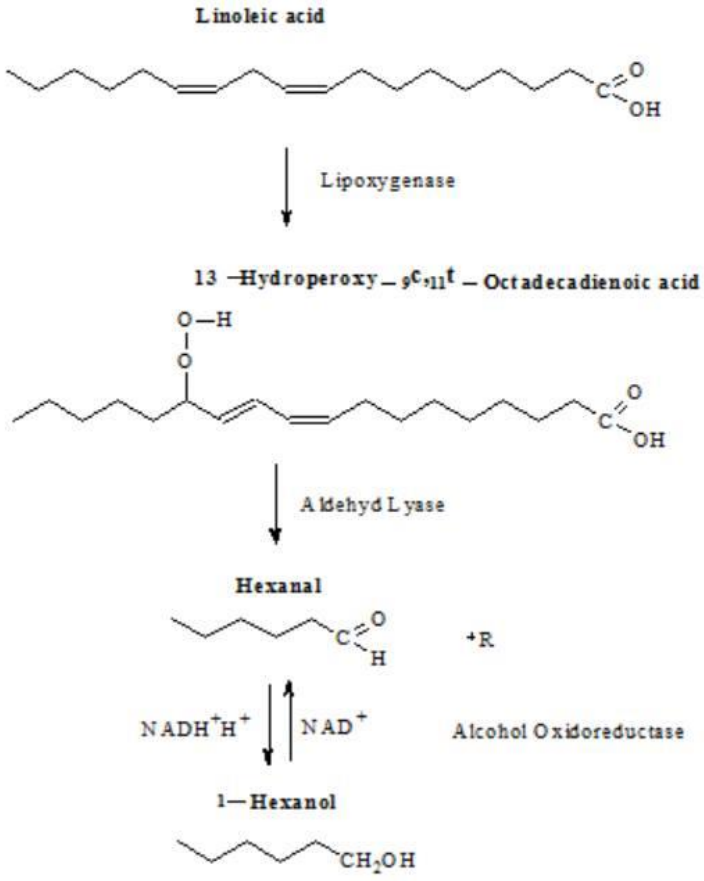

Figure 3: Genesis of hexanol due to oxidation of Linoleic acid. Described by Drawert (1974).

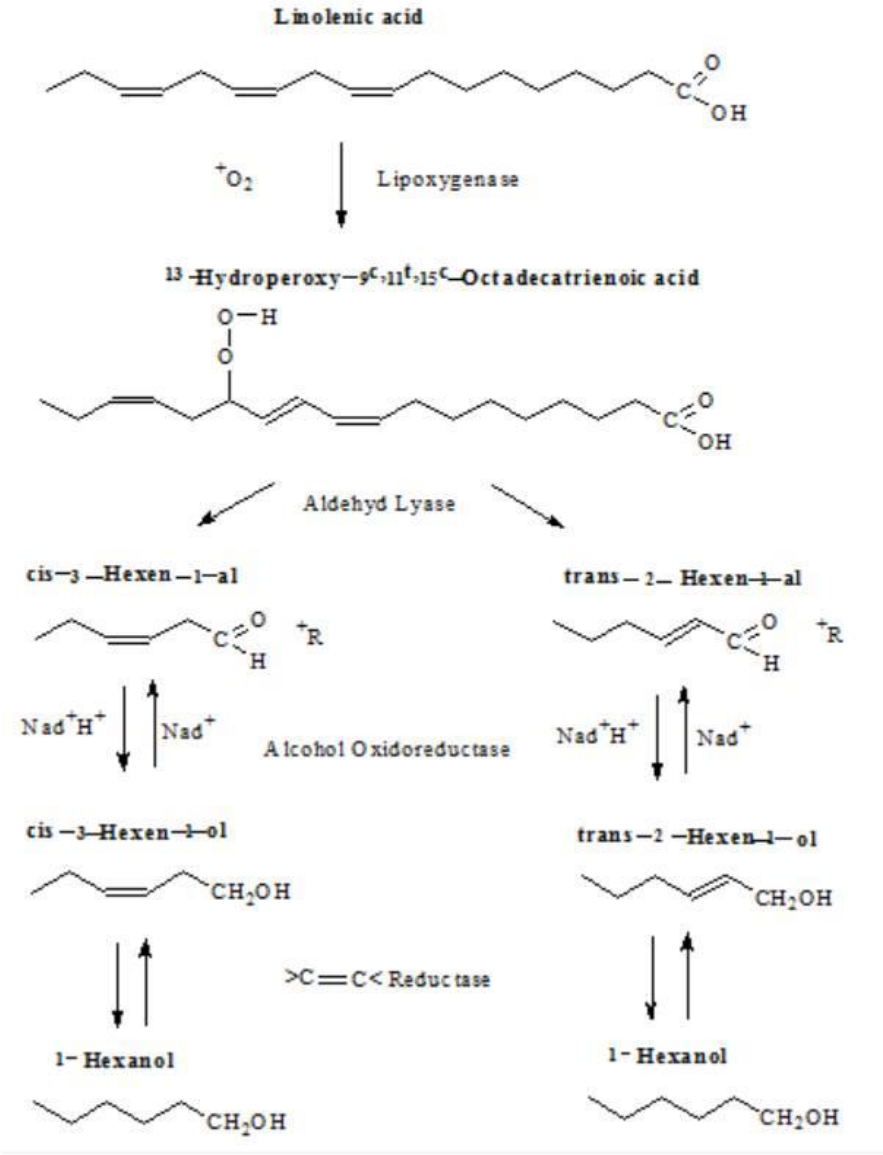

Figure 4: Genesis of hexanol due to oxidation of Linolenic acid. Described by Drawert (1974).

3.5 Inoculation rates:

Research on icewine has shown that an inoculation of 0.5 g/l had higher fermentation capacities than an inoculation of only 0.2 g/l. This may be due to higher biomass and cell concentrations attained at the 0.5 g/l inoculation rate (Kontkanem et al., 2004). Normally the initial yeast population doubles 5 times if grown in anaerobic conditions to reach a final population of approximately 120-150 × 10^6 cells/ml, depending on the yeast strain when 30-50 % of sugar remains to be fermented. The addition of 25 g/hl of dry yeast, after rehydration provides an initial cell density of approximately 5 × 10^6 cells/ml needed to achieve that goal but the author gives a value arising from personal experience of 3 × 10^6 cells/ml minimum,

which equates to approximately 15 g/hl as a minimum (Monk, 1997). Miller et al., (2007) have also shown that the addition of nitrogen sources, preferable NH_4 but also the addition of amino acids, increased fermentation capacities of yeast and made fermentations finish in a shorter time than control wines. Interesting in the research of Kontkanem et al., (2004) where yeast cells were shown to double more often at lower inoculation rates before going into a stationary phase. This result can be confirmed by the work of Lee et al., (2004) aswell. Here, even though initial inoculation rates were different by a factor of two, the average total cell count in between the treatments was less than 15 %. There are two major factors, limiting cell growth, being partly able to supplement each other: one of it is molecular oxygen, partly replaceable by two, fatty acids and sterol, which objective has been discussed before (Monk, 1997). This leads to the suggestion that yeast, inoculated at lower rates have to struggle more for the limited amount of these nutrients to double till the sufficient cell number. This stress will also have effects on metabolism and gene expression on some enzymes playing a role in aroma composition and maybe alcohol yield. Dried yeast cell cultures are known to have similar sterol reserves. This implements that a higher inoculation rate might have similar effects as the supplementation with fatty acids and sterols as cells can benefit of their lipid storages and finally do not have to divide that often to reach desirable cell numbers, which is known to dissolve lipid reserves between yeast generations (Deytieux et al., 2005).

Williams & Boulton, (1983) showed in a model that higher ethanol losses have to be expected from ferments of higher initial sugar concentration. Also higher inoculation rates have been suggested to produce higher ethanol evaporation due to higher fermentation rates and higher fermentation temperature, which finally greatly affects the transfer of ethanol into a gaseous phase. Joshi et al., (2013) decreased the final ethanol content in apple wine by addition of insoluble solids, previous to the fermentation. Although these results have not been confirmed statistically, they show a trend which may result from a decreased fermentability of must, caused by turbidity. Swiegers et al., (2006) suggested that warmer fermentation temperatures could also lead to thiols, formerly released during fermentation could get boiled off and be released from the ferment as part of the gaseous phase. Lee et al., (2004) could not show any effect on ethanol yield, arising from different inoculation rates either in a low (12.3 %) or a higher (14 %) alcohol degree ferment. It is also suggested that fermentation container design has an effect on final ethanol extraction out the container as ethanol may partly condensate in the headspace and be automatically reintroduced to the ferment. Different results have to be expected by red wine ferments in open top fermenters. It was shown that cap temperature could be up to 8-11°C higher than the liquid phase of the ferment and contains ethanol concentrations up to twice as high (Guymon & Crowell, 1977). Therefore, higher evaporation losses may have to be expected for red ferments. It was shown that higher concentration of esters like ethyl acetate, ethyl butyrate, isoamyl acetate, ethyl hexanoate, hexyl acetate, ethyl

octanoate, ethyl decanoate could be achieved at higher inoculation rate (0.5 g/l) compared to the lower inoculation rate (0.25 mg/l) when alcohol levels were low (Lee et al., 2004). Higher rates of turbidity at the other hand lead to a decrease in total esters in an apple wine ferment (Joshi et al., 2013). These observations correlated with an increase in fermentation speed, which is likely to boil of a certain number of esters in the gaseous phase.

3.6 Goals of this work:

In the further progress of this thesis, it will be tried to illuminate the development of thiols a little more. Turbidity is known for two main advantages. First to deliver UFAs and second, to help to release CO_2.

If CO_2 would not be released from a ferment it will in the worst case denaturise proteins and enzymes within the yeast cell. It is therefore likely that a higher release of CO_2 may protect enzymes and therefore higher thiols expression may occur (Spilimbergo et al., 2005).

Smith et al., (1996) reported on the effect of UFAs not only on cell wall composition but also on enzyme activity. Swiegers et al., (2005) reported alcohol acetyltransferase, the same enzyme responsible for the formation of the ester ethyl acetate to be responsible for the transformation of 3MH to 3MHA. UFAs are known to increase acetyltransferase activity (Duan et al., 2015). At the same time, most 3MH precursors are found in skin (Peyrot des Gachons, 2002). As turbidity consists mainly of skin debris, an increase of 3MH during fermentation at higher turbidities is expectable. Therefore, the effect of grape solids on thiol production during fermentation should be clarified. Further, interactions of nitrogen compounds on thiol production have been stated (Subileleau et al., 2008). But differences have been monitored between nitrogen sources and yeast strain, effects of different nitrogen sources in combination with turbidity shall be analysed more in advance as suggested by Tesnière et al., (2013).

4.0 Materials and methods:

4.1 Inoculation trial:

4.1.1 Initial juice:

Initially juice was collected from a commercial Marlborough winery in 2015 (further referred to as Marlborough juice), and stored at -1°C with FS of 40 ppm. To help maximise the aroma potential in the finished wines the concentration of green leaf volatiles was increased with the addition of freshly processed grape juice. Grapes (2.5 kg) of early maturity were harvested from OB vineyard where an irrigation regime of 3 weeks irrigation deficit was applied before veraison. Fruit was crushed in a manual crusher (Marchisio Cervino 400/600 kg/h) and rachis were removed by hand under reductive conditions. 2.5 l of stored Marlborough juice was added to the crushed and destemmed fruit and given 24 h skin contact at 6 °C. Fruit was then pressed in a compressed air operated 6 kg sample press (Stainless Steel Systems, Blenheim, New Zealand) under a cover of CO_2. The pressing regime consisted of depressing the plunger and pressing for one minute, releasing the plunger and stirring the marc. This regime was repeated three times. Pressed juice (1.6 l) was added to the stored Marlborough juice to make a final volume of 25 l. To decrease FS levels to <20 ppm for fermentation, two 80 ml additions of 0.3 % H_2O_2 was added at 30 minute intervals to the 25 l of juice. The final FS concentration before inoculation was 15 ppm (juice analysis found in attachment section)

4.1.2 Juice turbidity

Turbidity measurements were carried out, using a “Hach-2100Q IS” turbidity meter. Juice turbidity in the initial juice was 1.6 NTU following juice crossflow filtration after the 2015 harvest. After the addition of the freshly pressed juice, turbidity levels were as high as 65 NTU. Our target juice turbidity for this trial was a value between 100 and 150 NTU. Therefore “Laffort-Turbicel”, purified cellulose (C6H10O5), was added at a rate of 250 ppm to maintain juice neutral solids to help keep yeast in suspension. 0.25 g/l were diluted in 10 times its weight in water and added to the juice fraction. Final NTU readings of the juice before inoculation showed 126 NTU.

4.1.3 Yeast nutrition:

To provide sufficient nutrition to the ferments, “Beverage Supply Group-Superfood” (distributed by Pacific Rim Oenology, Blenheim, NZ) was added at two rates 300 mg/l and 600

mg/l directly to fermentation bottles. Juice was added later. “Superfood®” is a complex yeast nutrient, consisting of yeast hulls Nutrex 370. DAP (32.5 %), Yeast Extract T154, minerals and vitamins. “Superfood®” was chosen to provide yeast not only with Ammonia but also with compounds needed for cell wall building. NOPA, ammonia and YAN were analysed after additions ***(Table 1).***

Table 1 Initial YAN values

Sample	YAN (mg/l)
Single Superfood addition	216,4
Double Superfood addition	240.6

Yeast assimilable nitrogen (YAN) levels as found in single and double Superfood (SF) adjusted juices, before inoculation.

The values shown above result from two single measurements. “Superfood was actually chosen not only for its effect on YAN but for its nutrition complex, providing long term supplementation, not measurable in the analysis carried out. Therefore, Yan values as given above only show an indicator and were not used statistically. As given by The Australian Wine Research Institute, minimum YAN values are 150 mg/l, for a fruitier expression of wines, 250 mg/l to 350 mg/l are recommended. Both nutrition additions can be seen as sufficient for a non-sluggish or stuck fermentation.

4.1.4 Glutathione addition:

For antioxidative properties, food grade glutathione, produced by “Now Foods”(USA), was added to the juice. Glutathione comes in capsules of 433.7 mg of weight, containing 355.7 mg of powder. Each capsule contains 250 mg of Glutathione and fillers made of Rice Flour, Magnesium Stearate (vegetable source) and silica. A standard dose of 250 mg to 725 ml of juice is added at PFR. Glutathione was added to the initial base juice of 25l at the rate of 344.8 mg/l. Glutathione was added after desulfurization. Glutathione is a tripeptide consisting of glutamate, cysteine and glycine.

4.1.5 Repetitions:

Different Superfood doses were weighted and distributed straight into the fermenters. Each level of Superfood and yeast dosage was repeated three times. For four levels of yeast and 2

levels of Superfood, 24 fermenters were prepared. After Superfood was distributed, fermenters were filled under use of argon gas, up to a previous marked equal filling height. Finally, yeast which was previously weighted and rehydrated according to the PFR small scale winemaking protocol was added to the fermenters.

4.2 Turbidity trial:

4.2.1 Initial juice:

Juices used for the turbidity trial came from three different vineyards in the Marlborough region; TB, harvested on 5.4.2016, OB, harvested 6.4.2016 and GCL, harvested on 13.04.2016. The intention was to deliver an overall profile of Marlborough vineyards. Therefore, vineyards with different soils and different management techniques were chosen. TB, farmed organic (2057 vines/ha) yielded app.15T/ ha, GCL (2314 vines/ha) yielded app. 27T/ha and the OB (2314 vines/ha) yielded app. 20T/ha. In terms of vigour, the TB is slightly less vigorous as the OB. Both can be classified as low vigour sites whereas GCL is high vigour site.

Once the fruit parcels arrived at the winery, a 40 kg subsample of grapes was crushed and destemmed in an Enoitalia crusher/destemmer (Eno 1S, Italy). A standard sulphur dioxide (SO_2) addition (40 ppm) was added as potassium metabisulphate at crushing. A pectinase enzyme, Rapidase (rate 50 ml/t) was added to the crushed and destemmed fruit (must) and given one hour skin contact time before pressing. Grape must was pressed in a 20 kg hydro press (Marchisio, Italy) under a cover of CO_2. A pressing regime of 2 min. at 1 bar followed by 12 min at 3.5 bar was applied. An addition of bentonite Seporit PORE-TEC by ERBSLÖH (rate 0.5 g/l) was made to pressed juice. Juice was cold settled for 24 h at 10 ºC and then racked off juice lees. A juice sample was collected for analysis. A final volume of 17 l of settled juice was then set aside for winemaking per replicate.

4.2.2 Juice turbidity:

Figure 5. Different amounts of sediments as found after turbidity adjustment, before inoculation.

Juices pressed for the turbidity trial were handled according to the PFR winemaking regulations (to be found in the attachment section). After cold settling overnight, juices were racked, always by the same person to avoid variations in technique and lees composition. After the clear fraction was racked, the fluffy lees section was racked off the sediments, stored in 2 l jugs, and covered with argon gas till further processing. The clear juice fraction was further cut into 10 parts of 1.5 l and stored under argon cover in 4 l minibins. Then initial turbidity ***(Table 2)*** was adjusted by a multi-step process. Juice lees were added via a 10 ml pipette, and a 100 ml laboratory jug. After each addition, juices in the minibins were stirred, turbidity measured with the turbidity meter and argon gas added. This procedure was repeated till desired turbidity level was reached ***(Table 3)***. 2 minibins were kept as spare juices if turbidity level exceeded the desired level. After the juice turbidity adjustment, 0.7 l of the juice was bottled into the 0.75l fermenters ***(Figure 5)*** and inoculated under respect of the PFR small scale winemaking protocol, on the same day. This procedure was carried out for all three juice lots, ending in 3 fermenters for each turbidity level which was further seen as one repetition.

Table 2 Juice Turbidity

Origin/Vineyard	Tuamarina Block Replicate 1	Giffords Creek Lane Replicate 2	Omaka Block Replicate 3
Initial Turbidity	52.6 NTU	53.9 NTU	34.0 NTU

Initial juice turbidity given in Nephelometric Turbidity Unit (NTU), after racking, before adjustment of the desired turbidity levels.

Table 3 NTU levels after adjustment

Origin/ Vineyard	50 NTU Target	100 NTU Target	150 NTU Target	200 NTU Target	250 NTU Target	500 NTU Target	950 NTU Target	150 NTU-TC* Target
Tuamarina Block, Rep 1 Turbidity(NTU)	52.6	104	148	196	254	521	965	144
Derivation to target (%)	1.3	0.3	2.0	0.2	0.5	4.2	0.1	0.9
Giffords Creek Lane, Rep 2 Turbidity(NTU)	54.1	105	153	195	255	525	960	142
Derivation to target (%)	1.5	1.3	1.3	0.4	2.0	0.3	0.7	2.3
Omaka Block, Rep 3 Turbidity(NTU)	53.9	102	152	195	249	524	974	150
Derivation to target (%)	1.1	1.7	0.6	0.4	1.5	0.1	0.8	3.2
Total Average Turbidity(NTU)	53.3	103.7	151	195.7	252.7	523.3	966.3	145.3
Total Derivation to target (%)	0.8	1.5	2.7	0.6	3.2	2.1	7.1	4.2

Final turbidity levels given in Nephelometric Turbidity Unit (NTU), after adjustment with must lees. Derivation from the relevant average turbidity is given below each vineyard. A mean standard derivation is given in the last row.

*

4.2.3 Yeast nutrition:

Table 4 Initial nitrogen status

Vineyard/ origin	YAN (mg/l)
Tuamarina Block	141
Giffords Creek Lane	251
Omaka Block	88

Shows mean initial Yeast assimilable nitrogen (YAN) levels of the three vineyard blocks used within the turbidity trial, before adjustment with Diammonium Phosphate (DAP) and Superfood.

To maintain equal YAN values of 250 mg/l, “Superfood” and DAP was added to each bottle of ferment as required. As specified by the Plant and Food winemaking protocol, the required addition to achieve 250 mg/l total YAN was split, 50 % as delivered by DAP and 50 % as “Superfood”.

4.3 General procedure:

4.3.1 Bentonite:

Protein fining was carried out in both trials, using “Seporit PORE-TEC”-bentonite (ERBSLÒH) at a rate of 0.5 g/l to must. Additionally, prefermentative clarification was enhanced due to this step. “Seporit PORE-TEC” bentonite is known for its high purity and therefore releases almost no heavy metals to wine, during fermentation therefore no negative influence on thiols can be expected

4.3.2 Yeast inoculation:

All musts were inoculated with “Laffort-Zymaflore X5” commercial yeast, known for its thiol releasing properties. Standard procedure of inoculation at PFR is a standard yeast addition of 250 mg/l added to all 0.7 l treatments of the Turbidity trial. Yeast was rehydrated in 10 times of its weight in water and afterwards inoculated by pipette. For the Inoculation trial yeast was inoculated at a rate of 150 mg/l, 250 mg/l, 350 mg/l and 450 mg/l and followed the same PFR

yeast rehydration protocol as the turbidity trial. Temperature of the must at inoculation was 16 °C for both trials.

4.3.4 Yeast cell count:

Total yeast cell numbers were counted, using a "Hawksley-Counting chamber" (Haemocytometer) in style of an improved-Neubauer chamber. Cell counts were carried out, starting from 1.5 h after inoculation and on a daily base till 5 days after inoculation when cell counts reached a stationary phase. Dilution factors of 1.1 on the day of inoculation 2.2 on the first day after inoculation, 11-15 on the second day after inoculation and 100 from the third day after inoculation were used. Dilution was done, using distilled water. To make cells more visible 0.1 ml of "Aldrich-Methylene blue 0.05 wt.% solution in water was applied to each sample. Cell counts were further processed using the formula:

X=(d*n)/V where
X= total number of cells per ml
d= dilution factor
n= average number of cells per square
V= volume of square that was counted = $4*10^{-6}$

Equation 1: Shows the equation, applied to calculate yeast cells/ml.

Samples of 1 ml were taken after fermenters had been homogenized by mixing. Argon gas was applied before and after sample taking to avoid oxidation.

4.3.5 Total soluble solids and temperature measurements:

Total soluble solids (measured as °brix) and fermentation temperature were measured daily, using an "Anton Paar-DMA35" density meter (Anton Paar, Austria). Before and after measurements Argon gas was used to protect ferments and avoid oxidation. Once °Brix values dropped below 0 residual sugars were monitored daily using Clinitest® (Bayer, USA). When residual sugars reached 2.0 g l^{-1}, the ferments were stopped with the addition of 50 mg l^{-1} SO_2 (as potassium metabisulphite). Ferments were settled for 24 hours and wine was racked and centrifuged at 4600 rpm for 5 minutes using a Thermo Scientific Heraeus Multifuge 3SR+ centrifuge (Thermo Fisher Scientific) and samples taken for chemical analysis.

4.3.6 FTIR spectroscopy:

The infrared (IR) spectra of must samples (~35 ml) were obtained and analysed by a Foss Winescan FT2 instrument for the following parameters: °Brix, pH, total acidity, tartaric acid, malic acid, lactic acid, citric acid, ammonia, alpha amino acid N, glucose, fructose, potassium, ethanol, anthocyanins, Folin C Index, OD280 and OD520. The infrared (IR) spectra of wine samples (~35 ml) were obtained and analysed by a Foss Winescan FT2 instrument using the factory-set calibrations for the following parameters: pH, total acidity, tartaric acid, malic acid, lactic acid, glucose, fructose, ethanol, volatile acidity, reducing sugars, CO_2 and Folin C Index. The slope and intercept of the original Foss calibrations were modified using the previous season's data (vintage 2014 - 2015).
Sample acquisition was carried out in duplicate with a cuvette backflush, sample pre-flush of 3s, and intake time of 7s. An autoclean with Foss Winescan Cleaning agent (an aqueous hypochlorite solution) was carried out every 60s. IR spectra were obtained 956-5012cm^{-1} and reported Foss pin numbers from 240-1299 (*Wave number* (cm^{-1}) = 3.858× pin number). Thus each sample was analysed at 1009 individual wavenumbers. The areas of the spectra attributable to the absorption of water (PN400-445 and 770-940) were excluded.

4.3.7 Ethanol:

Alcohol was determined using an Anton Parr Wine Alcolyzer. All measurements were taken in duplicate with <0.02 v/v% variation.

4.3.8 Glucose and Fructose:

Glucose and fructose were quantified by enzymatic assay based on the reduction of NADP. All enzymes and cofactors were purchased from Megazyme. Samples were appropriately diluted and quantified in duplicate against an 8-point standard curve (R^2>0.98).

4.3.9 Yeast available nitrogen (YAN):

Ammonium was quantified by enzymatic assay monitoring the deprotonation of NADPH at 340 nm. All enzymes were purchased from Megazyme; ketoglutaric acid was purchased from Sigma Aldrich. Samples were appropriately diluted (usually two fold) and quantified in duplicate against and 5-point standard curve (R2>0.98). Primary amino acids were quantified in isoleucine (N) equivalents by the NOPA method adapted for the platereader.

4.3.10 Spectrophometry:

All spectrophotometric assays were run on a Molecular Devices Spectramax 384 Plus with a 1 cm pathlength cuvette reference correction. Optical density was measured directly in a UV transparent 96 well microplate at 280. 320. 420. 520 and 620 nm. Absorbance at 280 nm was used to quantify polyphenols against a gallic acid standard curve (5 point, $R^2 > 0.98$). All measurements were recorded in duplicate.

4.3.11 Organic acids:

Tartaric, malic, ascorbic, citric and succinic acids were quantified on a Shimadzu Prominence HPLC system using isocratic elution with a phosphate buffer (25mM, pH 2.5) on an Allure Organic Acids Restek column (5 µm, 240 x 4.6mm). All samples were diluted five fold in a solution containing oxalic acid as an internal standard and filtered through a 0.45 µm syringe filter prior to injection. All samples were run in duplicate and quantified on a five point standard curve (R^2>0.98).

4.3.12 Brix, titrable acidity and pH:

Titrable acidity and pH were determined on a Mettler Toledo T70 autotitrator using an equivalence point titration. Aqueous sodium hydroxide (0.1M) was used as titrant. °Brix was determined on a Mettler Toledo RM40 refractometer. Wine samples were degassed prior to analysis.

4.3.13 Thiol analysis method:

Analysis of the volatile thiols 3-MH, 3-MHA, and 4-MMP, was undertaken by Hill Laboratories in Hamilton, New Zealand. The method used for this commercial service is fully described by Green, et al., (2011). In summary wine volatile thiols were analysed by HS-SPME (Head Space Solid-Phase Micro-Extraction) with GC-MS/MS (Gas Chromatography-Two Dimensional Mass Spectrometry). Wine sub-samples were pipetted into 10 ml headspace vials, with NaCl added to “salt-out” thiols into the headspace. Vials were capped, and then deuterated internal standards for 3-MHA and 3-MH added to each vial, robotically, through the cap septa. Each sample was buffered to pH 6.5–7.0 with phosphate buffer before analysis, to avoid losses of thiols. Samples and calibration standards were extracted by SPME (polyacrylate, 85 µm coating supplied by Supelco Bellefonte, PA, USA), using a robotic CTC CombiPal auto-sampler (Agilent Technologies, CA, USA). For analysis, the SPME fibre was inserted into the hot (270 ˚C) GC inlet to desorb extracted thiols. Injections were in splitless

mode, with a 0.75 mm i.d. glass liner (Restek, PA, USA), at a temperature of 270 °C. Thiols were separated from co-extracted wine volatiles, on a HP-5MS capillary column (30 m×0.25 mm i.d.×0.25 µm film thickness, Agilent Technologies, CA, USA). Desorption of the SPME fibre was at a column oven temperature of 40 °C for 1 min, then progressively increased to 250 °C at a rate of 20 °C min−1. Helium carrier gas was set to a constant flow velocity of 1.5 ml min−1. Detection was by triple quadrupole mass spectrometry with an electron ionisation source (Agilent 7000B MS instrument, Agilent Technologies, CA, USA), using two multiple reaction monitoring (MRM) transitions per analyte (4-MMP:75/41, 89/55; 3-MH:134/82, 100/82; 3-MHA:116/88, 88/59) to give positive identification of peaks.

4.4 Statistics:

For the inoculation trial Analysis of variance and multiple comparisons by Fisher's protected least significant difference test were carried out. For the turbidity trial Analysis of variance and by Fisher's protected least significant difference test was carried out under respect of vineyards as block structure and fermentation time as a covariate.

All analysis of variance and Fisher's protected least significant difference test were done under usage of “GenStat”,17.th version, 17.1.0.14713.

All Graphs, were calculated under usage of “Microsoft Office Professional Plus 2013, Excel” The Logistic curve was calculated with help of Sigma Plot and further drawn with Microsoft Excel.

5.0 Results and Discussion:

5.1 Inoculation trial:

5.1.1 Fermentation kinetics:

No juice analysis has been carried out after the trial has been set up and before inoculation. The first measurements of density (°Brix) and temperature (°C) were taken 12 h after inoculation. As all juice derived from the same initial lot and only differed within yeast and Superfood addition, I consider initial °Brix to be the same in all bottles. As visible in ***Figure 6*** °Brix values were still narrowly distributed on the first day of fermentation. At the second measurement, on the second day after inoculation, all °Brix values differed statistically from each other. This status remained until the end of fermentation. Further, the biggest differences between data points existed during the first two days of fermentation, whereas the measurements get closer at the end of fermentation, as fermentation rates slow down. At the second day of measurements, the difference between the fastest and the slowest ferment was 2.66 °Brix. Differences attained at this point of fermentation, effected fermentation time of each treatment and can therefore be seen as the main source of difference in this trial.

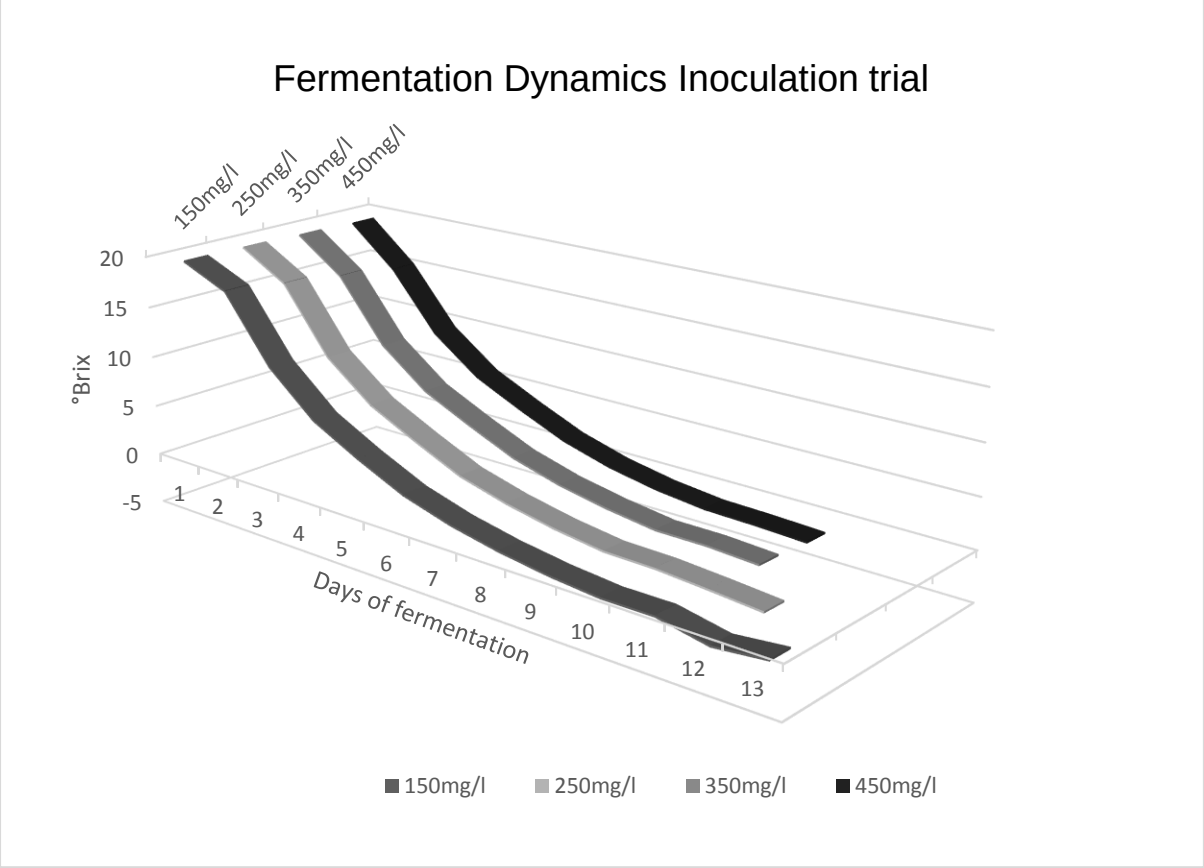

Figure 6. Fermentation dynamics for different inoculation levels

This implies that the greatest effect of yeast inoculation rate is found in the first 48 hours of fermentation, during the lag and exponential phase, before the stationary phase has been reached. These findings are expectable as initial biomass was three times as high in the highest inoculation dose when compared to the lowest inoculation dose. Yeast is therefore able reach the stationary phase earlier and to ferment bigger quantities of sugars earlier during the fermentation, which explains occurring differences. Once the media starts to be depleted of substrates, required for further growth, and a maximum biomass has been reached, the behaviour of the different ferments becomes more equal. All ferments fermented to total dryness.

Table 5 Fermentation times

Treatment	150 mg/l Yeast dosage	250 mg/l Yeast dosage	350 mg/l Yeast dosage	450 mg/l Yeast dosage
Single Superfood addition	13	12	11	11
Double Superfood addition	10	9	9	9

Fermentation time (days) of different inoculation levels and Superfood (SF) doses as applied in the inoculation trial.

At the time of the first measurement of °Brix, a significant (P=<.001) effect on fermentation time as measured in days for yeast inoculation rate was measured. When Fisher's protected least significant difference test was carried out, no difference between an inoculation of 150 mg/l and 250 mg/l could be found, but there was a reduction in °Brix for 350 mg/l and 450 mg/l of 0.17 and 0.28 °Brix respectively. On the second day of inoculation, difference in °Brix Increased up to 2.29 °Brix between the 150 mg and the 450 mg inoculation. From the second day of fermentation till the 10th day of fermentation, significant effects of inoculation rate (<.001) and Superfood (<.001) were identified, but none overcame differences as seen at the second day of measurements. During this time, all levels of yeast inoculation, differed from each other. No further analysis of variance was carried out from day 10 of fermentation as first trials completed their fermentation. To clarify the differences in fermentation kinetics a logistic graph

has been created. Logistic curves are widely used to describe population within a logarithmic S shaped function.

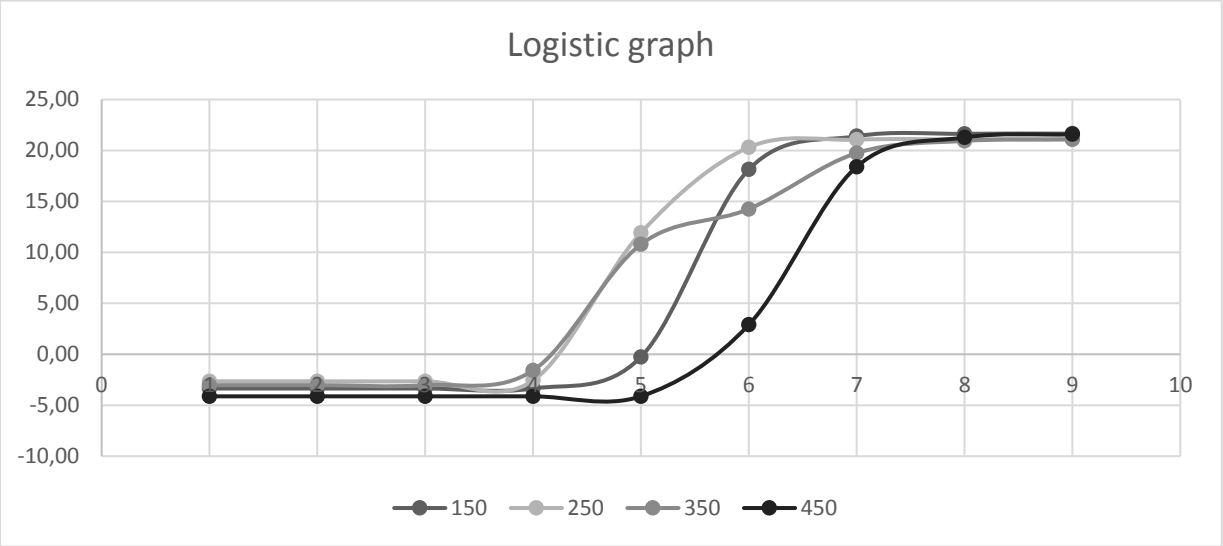

Figure 7. Logistic curve for all four inoculation levels (mg/l), including Superfood doses.

The Logistic curve was not sufficient to draw clear conclusions about the fermentation kinetics of the trial. The single curves didn't follow the expected pattern, seen before in ***Figure 6***. The curve made differences visible at onset of fermentation and the actual speed of fermentation, as shown in the curves inclination.

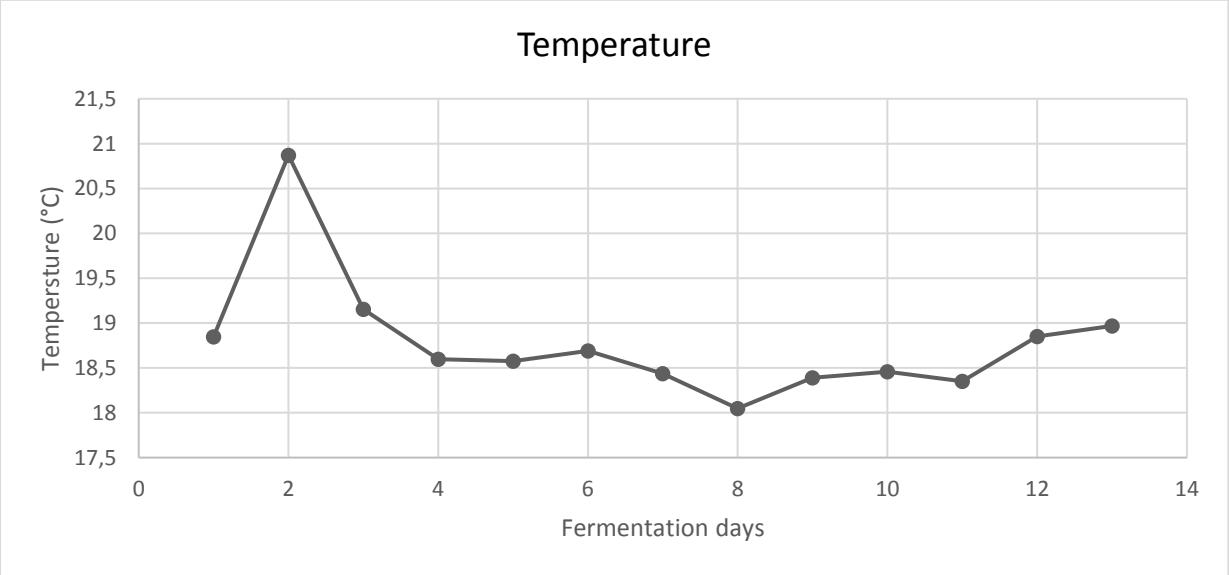

Figure 8. Fermentation temperatures as recorded during the inoculation trial.

For the Inoculation trial temperature was targeted at 18°C. The cooling system, which is applied indirectly, chilling down the whole fermentation room, had some mechanical issues during the first 48 h of fermentation, which lead to an increase in temperature (20.87 °C). The mean fermentation temperature during the fermentation trial was 18.78 °C. This rise in temperature during the first 48 h may have sped up yeast metabolism, especially on the second day of fermentation and influenced results reported in the following section.

Table 6 Table of means and statistical relevance of wines derived from the inoculation trial.

Values	150	250	350	450	P	Superfood*1	Superfood*2	P	Interaction
A420/A320 (nm) *	**0.025 bc**	**0.026 c**	**0.024 ab**	**0.023 a**	**0.01**	**0.026**	**0.023**	**0.009**	0.053
Tartaric acid (g/l)	**4.3 a**	**4.4 b**	**4.5 b**	**4.8 c**	**<.001**	**4.411**	**4.588**	**<.001**	**<.001**
Malic acid (g/l)	**4.0 a**	**4.2 b**	**4.7 c**	**4.8 c**	**<.001**	**4.4**	**4.5**	**0.04**	**<.001**
Ascorbic acid (g/l)	**0.19 a**	**0.19 a**	**0.21 b**	**0.22 c**	**<.001**	0.20	0.20	0.299	0.268
Acetic acid (g/l)	0.92	0.89	0.80	0.87	0.267	**0.94**	**0.81**	**0.009**	**0.014**
Succinic acid (g/l)	**0.80 a**	**0.91 b**	**1.15 c**	**1.22 c**	**<.001**	1.01	1.03	0.698	**<.001**
pH	**3.10 c**	**3.08 b**	**3.07 ab**	**3.06 a**	**<.001**	**3.10**	**3.05**	**<.001**	**<.001**
Titratable acidity (g/l)	**9.5 a**	**9.7 a**	**9.6 a**	**10.0 b**	**0.012**	9.7	9.7	0.711	**0.002**
Reducing sugars (g/l)	**0.9 c**	**0.8 c**	**0.6 b**	**0.1 a**	**<.001**	**1.1**	**0.1**	**<.001**	**<.001**
Folin Ciocalteu ***	**511 c**	**480 b**	**451 a**	**444 a**	**<.001**	**483**	**460**	**<.001**	0.132
Polyphenols (mg/l GAE) **	351	349	345	350	0.084	348	349	0.470	**<.001**
Alcohol corrected (%) ****	**12.0 d**	**11.8 c**	**11.7 b**	**11.7 a**	**<.001**	**11.9**	**11.6**	**<.001**	**<.001**

Means followed by different letters within a row are significantly different at the least significant difference level of 5 % (Fischers protected LSD)

* ***A420/A320 is an oxidative index***

** ***GAE = Gallic acid equivalents***

*** ***Folin Ciocalteu = Winescan derived data***

**** ***Alcohol, after calculative conversion of reducing Sugars***

5.1.2 General results:

Inoculation rates had effects on pH values ***(Table 6)***. Inoculation with 450 mg/l yeast showed significant difference (P=<.001) to the 250 and 150 mg/l yeast rate. Further significant differences (P=<.001) were detected between the 350 mg/l and 150 mg/l inoculation rate aswell as between the 250 mg/l and the 150 mg/l inoculation rate. Concluding, the higher the amount of inoculated yeast, the lower the pH. Differences varied between 0.017 and 0.04 units. The use of the higher Superfood dose lead to a pH value, significant (P=<.001) 0.043 units lower than the single Superfood addition. Finally, a significant (P=<.001) interaction between the amount of Superfood added and the yeast amount inoculated was detected.

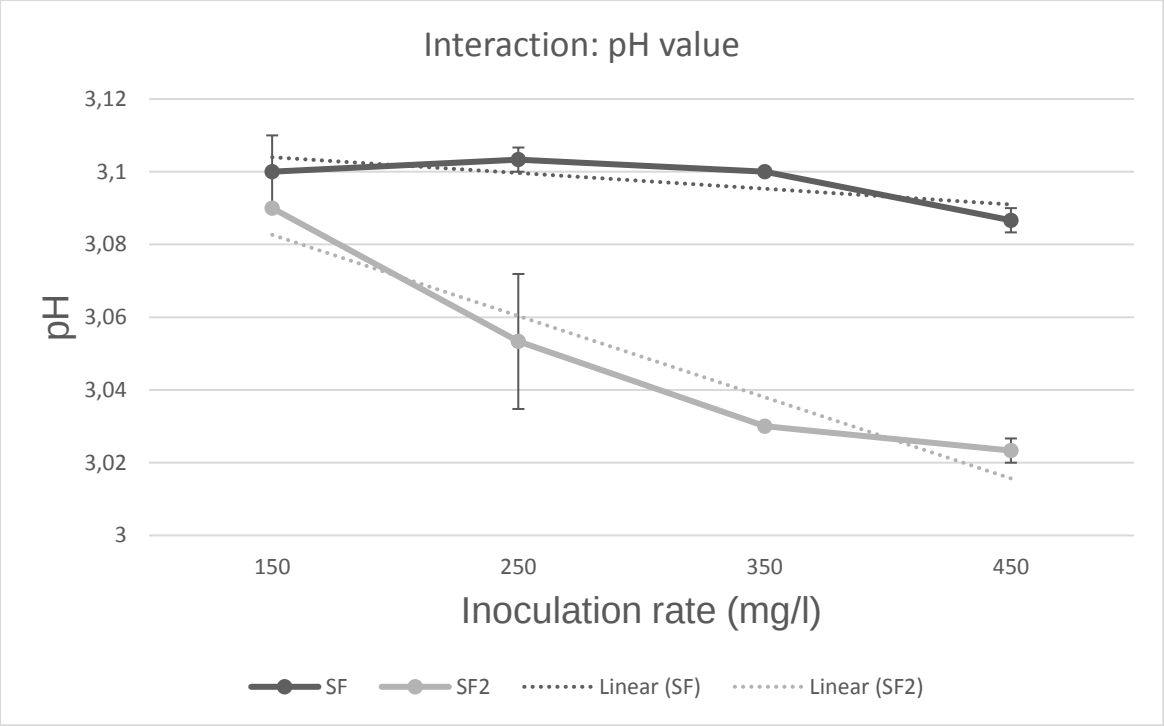

Figure 9 Interactions between the single and the double Superfood dosage as found for pH values within the Inoculation trial plus trendlines and error bars

Taking a closer look at the interactions found to be significant (P=<.001) during analysis of pH, it occurs that at an inoculation rate, of 150 mg/l, both the SF and SF2 dataset don't differ statistically. Further, main effects monitored during the analysis only apply for SF2, which the trendlines reveal.

Titratable acidity (g/l) was significant (P=0.012) affected by the dosage level of yeast ***(Table 6)***. The inoculation of 450 mg/l of yeast had higher total acidity as all other inoculation rates. No difference was detected between the 150, 250 and 350 mg/l inoculation rate, although titratable acidity trended to be lower at lower inoculation rates. The difference between the highest and the lowest amount of total acidity was 0.478 g/l. No effect of the Superfood addition

to titratable acidity was detected. Results showed a significant (P=<.001) interaction effect of Superfood and the inoculation rate on titratable acidity.

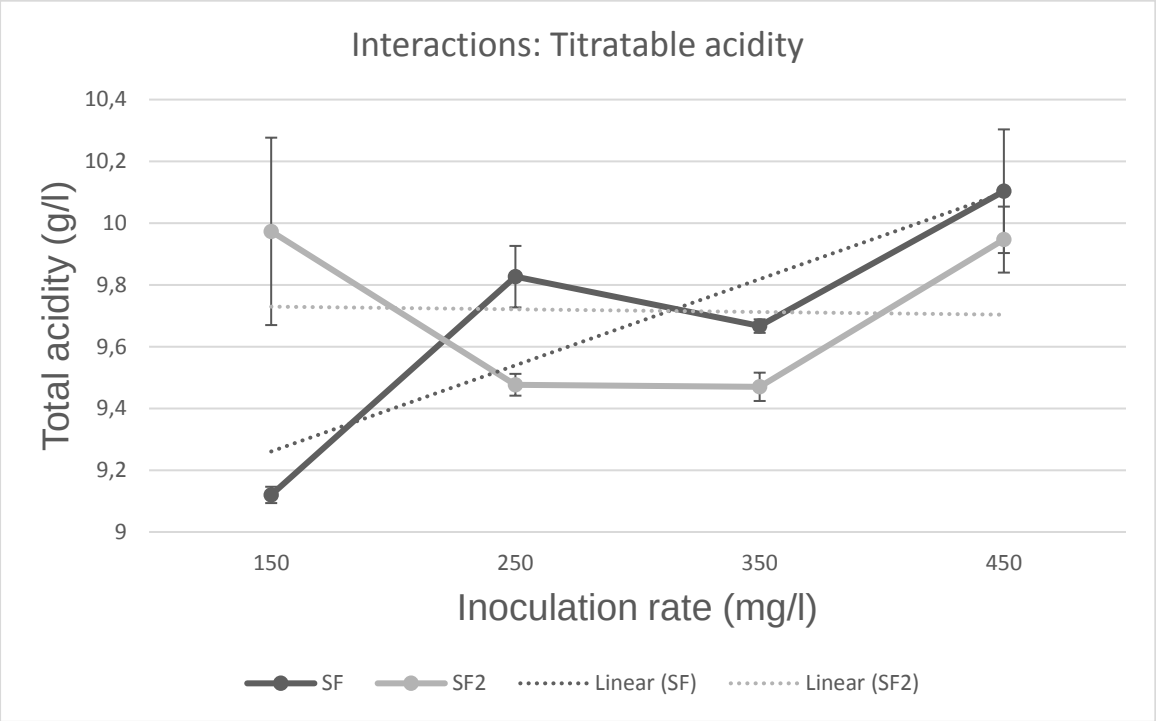

Figure 10 Interactions between the single and the double Superfood dosage as found for titratable acidity (g/l) values within the Inoculation trial plus trendlines and error bars

Closer observation of the interactions found during analysis reveal that statistical difference was mainly caused by SF. The trendline of the SF2 dataset continues almost horizontal. Main reason for this trend is the high acidity detected at 150 mg of yeast inoculated within this dataset. The values measured at 450 mg/l inoculation rate do not differ between the two Superfood dosages. Overall conclusions extracted from this analysis have to be rated carefully and further research has to be carried out to clarify results.

As these trends were also seen during Winescan analysis, further analysis of organic acids was carried out. Results showed significant (P=<.001) higher degradation of malic acid at lower inoculation rates if compared to higher inoculation rates ***(Table 6)***. SF aswell as SF2 showed differences between every step of inoculation. The only exception was SF, where no difference could be found between an inoculation of 250 and 350 mg/l.

This degradation is most likely due to the phenomenon of maloethanolic fermentation (MEF) which is the degradation of malic acid to ethanol during alcoholic fermentation. Malate degradation is part of both, the aerobic and anaerobic part of the TCA cycle which takes place in the mitochondria under aerobic conditions where it is transferred via the malic enzyme to ethanol and in the cytosol under anaerobic conditions, where it is transferred via fumarase to succinate (Boles 1998; Redzepovic et al., 2002, Saayman & Viljoen-Bloom, 2006). Changes

in malic acid and ethanol content have been monitored in apple wine before by Joshy et al., (2013). The ability to metabolize extracellular malic acid is dependent on the uptake system of malic acid into the cell and further the activity of the malic enzyme. The malic enzyme is responsible for the conversion of malic acid into pyruvic acid via oxidative decarboxylation with the further endproducts CO_2 and NAD(P)H. Pyruvic acid as an important metabolite within carbohydrate metabolism can later be further be decarboxylised to acetaldehyde by pyruvate decarboxylase and finally be reduced to ethanol and CO_2 by alcohol dehydrogenase (Vollschenk et al., 2003). *Saccharomyces cerevisiae* is widely known for its poor malate usage. The uptake of extracellular malate aswell as other dicarboxylic acids is possible for cells of this genus only by simple diffusion (Salmon., 1987). Therefore, an increase in malic acid degradation can be correlated with initial malic acid concentration (Delcourt et al., 1995). Still, there exist strain dependent differences within *saccharomyces* strains in the ability to degrade malic acid (Volschenk et al., 2003). Studies by Redzepovic et al., (2003) revealed the ability of *saccharomyces cerevisiae* (strain Lalvin 71B) to reduce 18 % of malic acid during fermentation. Yeast can be divided into two groups according to their ability to use malate and other tricarboxylic acids as a sole source of energy K(+). *Saccharomyces* are classified as K (-) as they are only able to utilize TCA cycle intermediates in the presence of glucose or other assimilable carbon sources. Additionally, the malic enzyme of *saccharomyces cerevisiae* shows a very low substrate affinity (Temperli et al.,1965). Main genes involved in malic acid metabolism of yeast are MAE1 and MAE2. Boles et al., (1998) showed relative low expression of MAE1, the gene involved in malic enzyme expression. It was further proved that the survival of *saccharomyces cerevisiae* is not dependent on the malic acid enzyme.

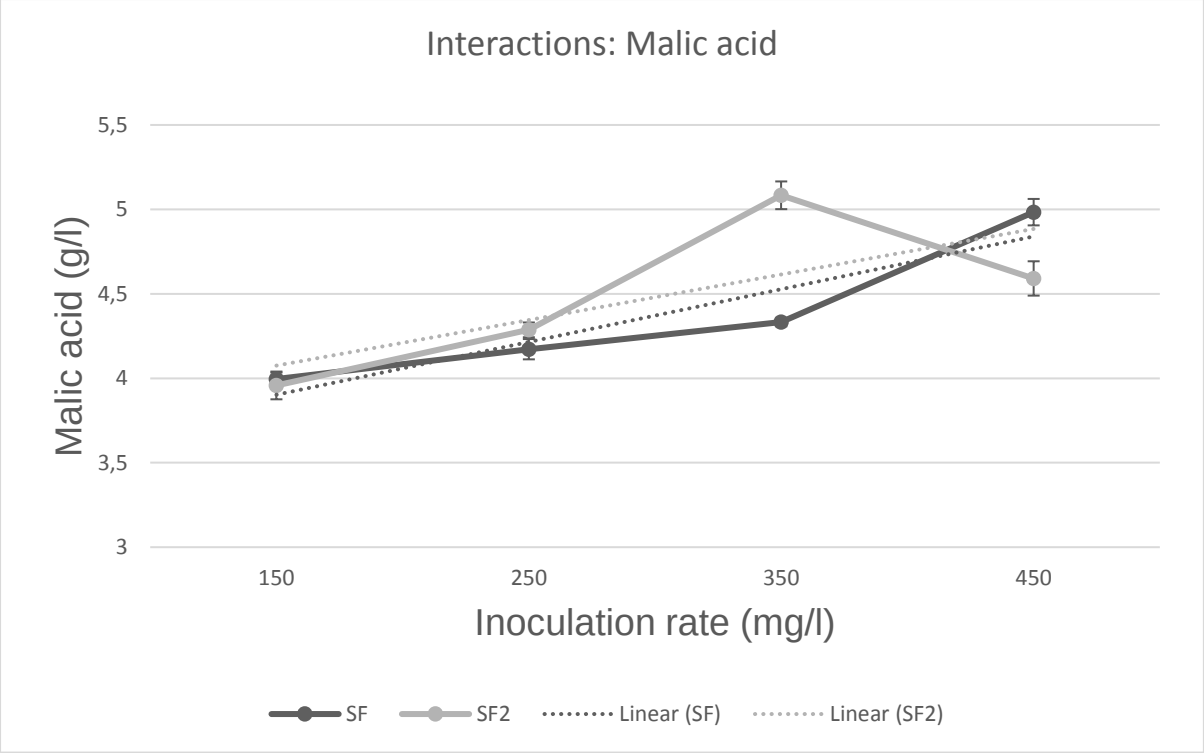

Figure 11 Interactions between the single and the double Superfood dosage as found for malic acid (g/l) values within the Inoculation trial plus trendlines and error bars

Due to significant (P=<.001) interactions within the malic acid analysis, a closer look was taken. It was seen that only very minor differences occurred between the single and the double Superfood addition. An overall trend towards higher malic acid at higher inoculation doses was seen though. Differences between 350 and 450 mg/l yeast inoculation within the SF2 dataset are hard to explain and should be considered if conclusions obtained from this analysis are drawn. Main value to point out is the 450 mg/l inoculation dose within the SF2 dataset which shows lower malic acid vales than the 350 mg/l inoculation dose. A continuous degradation of malic acid was therefore only monitored within the SF dataset.

Findings of ethanol yield correlated with the findings in malic acid and underline the theory of MEF. Ethanol yield was significant (P=<.001) affected by the amount of yeast cells inoculated ***(Table 6)***. The higher the amount of yeast cells inoculated, the lower the alcohol yield. Significant differences were detected between 150, 250 and 350 mg/l aswell as between 150, 250 and 450 mg/l of yeast inoculated. No differences were detected between the inoculation rates 350 and 450 mg/l. The difference between the highest and the lowest inoculation rate was 0.24 % of ethanol. Also Superfood had a significant effect (P=<.001) on ethanol yield. The difference between the higher yielding SF2 and the lower yielding SF was 0.2271 %.

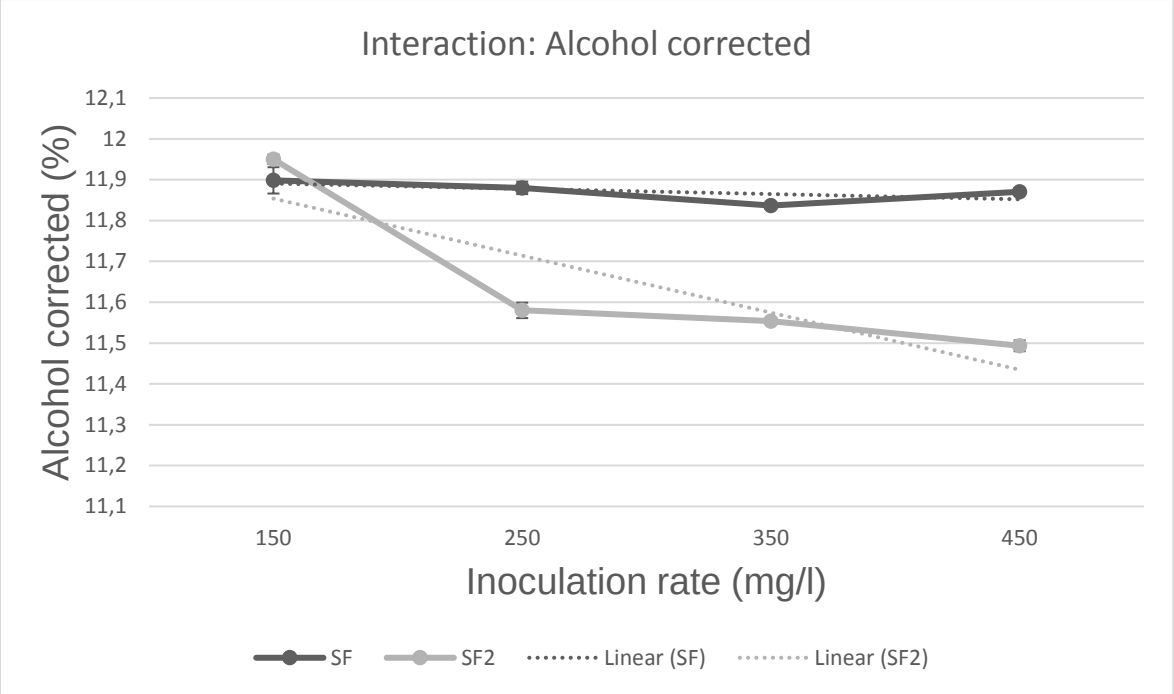

Figure 12 Interactions between the single and the double Superfood dosage as found for alcohol corrected (%) values within the Inoculation trial plus trendlines and error bars

Interactions found within ethanol analysis show only a minor trend towards lower ethanol levels within the SF dataset. The main effect found to be significant during the statistical analysis is caused by changes in the SF2 dataset. Further ethanol level at 150 mg inoculation seem to equal for both datasets.

Significant differences (P=<.001) have been monitored within tartaric acid amounts found during the analysis of organic acids ***(Table 6).*** A clear trend was seen for lower inoculation rates to have lower amounts of tartaric acid. Difference between the lowest and the highest inoculation rate was 0.461 g/l. Further a significant effect (P=<.001) of Superfood was seen. Higher superfood dosage ended in higher levels of tartaric acid. Finally, an interaction (P=<.001) was found to occur for tartaric acid.

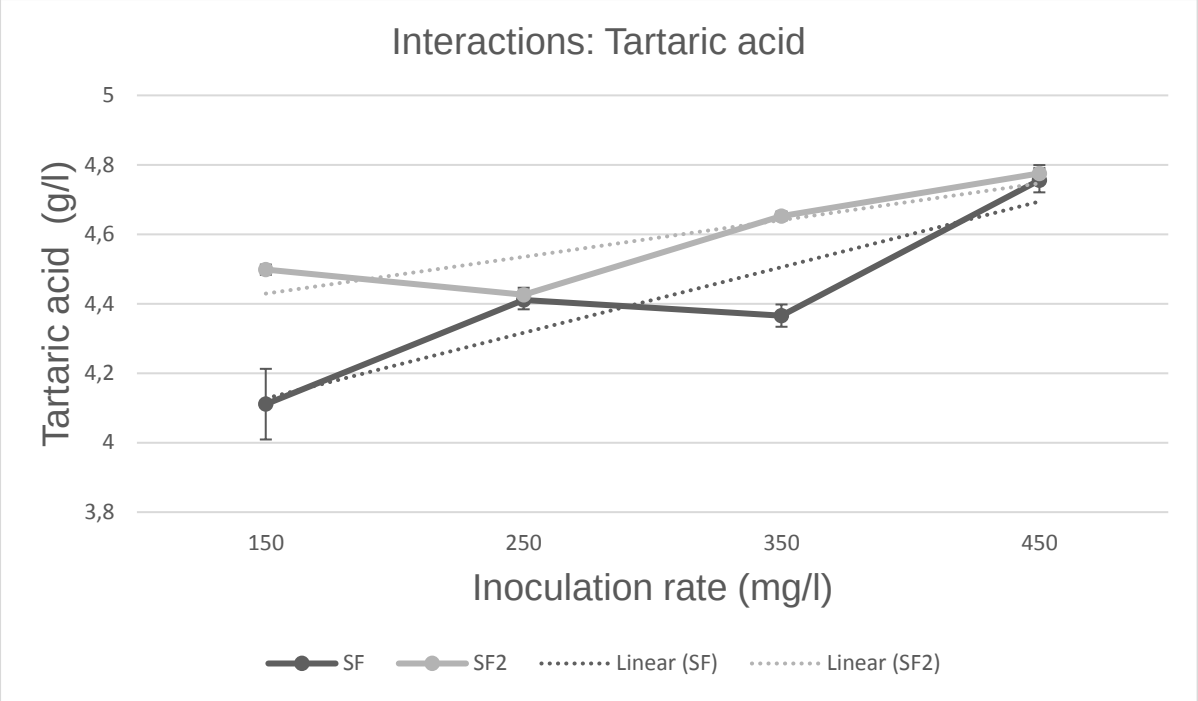

Figure 13 Interactions between the single and the double Superfood dosage as found for tartaric acid (g/l) values within the Inoculation trial plus trendlines and error bars

A closer look at interactions for the analysis of tartaric acid reveals a trend towards higher tartaric acidity levels at higher inoculation rates. Still, conclusions are hard to draw according to this results as in the SF dataset, the value of 250 mg/l shows higher tartaric acid as the value for 350 mg/l. Also in the SF2 dataset some parts are hard to rate. The 250mg ferment showed lower tartaric acid values than the 150 mg inoculation. Finally, the values of both datasets are equal at a dosage of 250 mg/l of yeast.

Results need to be interpreted with caution as samples have been frozen before analysis of organic acids, which is known to limit solubility of tartrates and forces them to precipitate. However, tartrate precipitation normally is a long lasting effect and as our samples have been treated the same way, it is unlikely that statistical differences only occur due to the process of freezing. Tartaric acid is along with malic acid one of the main acids found in grape juice. Very few microorganisms are capable of degrading tartaric acid (Ribereau-Gayon, 2006). Tartaric acid is likely to form salts in combination with calcium and potassium, namely potassium hydrogen tartrate (KHT) and calcium tartrate (Jackson et al., 2008). This phenomenon is dependent on ethanol content, pH, temperature, presence of other cations and anions, and proteins (Postel et al., 1984). To stabilize wines in terms of tartrate crystallization, 3 general ways of processing are used: 1.) processes that induce salt precipitation like the cooling of wine and the introduction of crystallization points. 2.) Ion exchange processes that reduce the amount of calcium and or potassium ions. 3.) Processes that inhibit crystallization (Gerbaud et al.,2010). Compounds used to inhibit crystallization or modify their solubility at lower

temperatures are metatartaric acid, carboxymethylcellulose (CMC) and mannoproteins (Lasanta & Gomez., 2012). Mannoproteins are glycoproteins, polysaccharides part of the cell wall of *saccharomyces cerevisiae*. They are released during fermentation, to a higher content during ageing on lees due to autolysis or can even be added directly to wine (Laubers et al., 1987; Boissou et al., 2007). According to Gerbaud et al., (1996), mannoproteins bind to nucleation points and therefore stop crystal growth. In the inoculation trial, several factors may have played a role in tartrate precipitation. First, higher ethanol levels within lower inoculation rates may have forced higher tartrate crystallisation. Secondly both higher inoculation rate and the introduction of yeast hulls Nutrex 370 within the addition of Superfood may have had an impact on the release of crystallization inhibitory polysaccharides. Fernandez et al., (2005) have reported the release of mannoproteins from yeast cell hulls previously. Laubers et al., (1987) showed that the release of polysaccharides from yeast cells depends on yeast strain, fermentation temperature, duration of storage on lees. The stirring of lees, was beneficial for the release of mannoproteins. Stirring of the fermenters as applied to homogenize samples for yeast cell counts may have increased the release of mannoproteins additionally.

Salmon, (2005) pointed out the high antioxidative capacities of wine lees, which stay active at least as long as 3 years of lees ageing. Vivas et al., (2000) proved that components released during yeast autolysis can further slowdown oxidation. A significant effect (P=0.01) of yeast inoculation rate on A420/A320 ratio was detected. A420/A320 ratio is described as an index of oxidation, which includes total hydrocinnamates as measured by A320 and browning of wine as measured within A420 analysis (Allen et al., 2011). Inoculation rate of 450 mg/l of yeast had lowest A420/A320 ratio ***(Table 6).*** Inoculation rate of 250 mg/l had the highest rate, being 0.00387 units higher than the inoculation of 450 mg/l of yeast. The difference between 450 and 150 mg/l was only 0.00247. No difference was detected between 350 and 150 mg/l. No differences were found between 150 and 250 mg/l. A higher addition of Superfood lead to a lower A420/A320 ratio. The difference SF and SF2 was 0.00211 units. No interaction between Superfood and the yeast dosage was detected.

A trend of low inoculation rates showing a higher Folin Ciocalteu index than lower ones, was shown during this research. No difference was detected between the 450 and 350 mg/l yeast dosage. Significant differences (P=<.001) were shown to occur between 250, 150 and 450 mg/l aswell as between 250, 150 and 350 mg/l. The difference between the highest and the lowest Folin Ciocalteu rate was 64.4 units. Also the amount of Superfood added showed a significant effect (P=<.001) on Folin Ciocalteu index, giving SF2 a 22.8 units lower value than SF. The difference between SF and SF2 was 22.8 units. No interaction between Superfood and the yeast dosage was detected. The Folin Ciocalteu method describes a measurement of all phenolic substances within a plant extract and its usage is widely accepted in wine. Besides

phenols, reducing substances are detected aswell. That's why this index is used to describe the total reducing capacity of a sample. Values obtained during this trial are hard to rate as significant differences of reducing sugars and ascorbic acid occurred, which are known interferences of the Folin Ciocalteu method, and their interaction is unknown (Waterhouse A.L., 2002). Further Proteins composition is known to interfere with the Folin Ciocalteu method. Different protein composition although not measured, are expectable due to the introduction of different yeast and nutrient doses.

During fermentation phenolic content of wines increased in average by 166.15 mg/l. No significance was monitored during fermentation between the different yeast or superfood additions. Reason for this increase during fermentation can only be generated by a breakdown of skin debris added within the fresh pressed juice which aimed to increase green leaf volatiles in the samples. This is an expected result as white wines, elaborated without maceration are generally low in polyphenols. Also interaction between yeast and polyphenols was to my best knowledge not reported so far and cannot be used as an alternate hypothesis.

Ascorbic acid normally only occurs in small doses (around 50 mg/l) naturally in grape juices. It doesn't occur to the extent it was found during organic acid analysis in the inoculation trial. I therefore assume that ascorbic acid has been added during the grape processing at the winery from where juice was bought originally. As all juices were separated from the same initial lot, the decrease of ascorbic acid during fermentation is interesting. A significant effect (P=<.001) of the inoculation rate on the amount of ascorbic acid was shown during the trial ***(Table 6)***. The decrease of initial ascorbic acid was higher at lower inoculation rates. The difference between the 150 mg and the 450 mg inoculation rate was 0.0355 g/l. Ascorbic acid is a widely used additive in winemaking, used to prevent oxidation and reduce the use of SO_2. Further, research has revealed that addition of high doses of ascorbic acid in combination with SO_2 to machine harvested fruit, can increase the amount of varietal thiols produced during fermentation (Grose et al., 2013). In New Zealand, the addition of 250 mg/l of ascorbic acid is allowed by law, the amount found in the final wine shall not exceed 300 mg/l. Ascorbic acid reacts in a metal ion mediated reaction with molecular oxygen and forms thereby H_2O_2 and dehydroascorbic acid. These compounds then react with SO_2 and form H_2SO_4 in the case of H_2O_2 or form a reversible bind in the case of dehydroascorbic acid (Barril et al., 2016). This makes it obvious that the use of ascorbic acid is only recommended with sufficient levels of SO_2 to scavenge H_2O_2 radicals, which could otherwise oxidise the wine varietal thiols directly or over hydrocinnamic acid released quinones (Blanchard et al., 2004; Nikolantonaki & Waterhouse, 2012). During fermentation, FS is usually bound quickly to carbonyl compounds, mainly ethanol (acetaldehyde), produced by yeast to protect itself from the antiseptic effect of SO_2 (Ribereau-Gayon et al., 2006). Research recommended different levels of free SO_2 when

ascorbic acid is used to avoid its oxidative effect, but minimum levels of 10ppm seem to be crucial (Barril et al., 2016). Nikolantonaki et al., (2014) gave values of 20 ppm to best protect varietal thiols. During the inoculation trial, free SO_2 was reduced by H_2O_2 to 15 ppm previous to fermentation. SO_2 is thereby oxidised into H_2SO_4 (sulfuric acid), leaving the remaining wine with rather little SO_2 protection. During fermentation, grape juice is known to be in a stage of reduction. Introduced oxygen is used immediately by yeast cells for the synthesis of sterols and fatty acids. The antioxidative effect may be even bigger when higher yeast cell mass is present as suspected by the 450 mg/l inoculation level.

Ascorbic acid values only correlated to a lesser extent with fermentation time (R^2=0.3346). Therefore, the main effect of higher inoculation doses is to be found in the faster onset of fermentation as seen in both, SF as well as SF2. Higher yeast cell biomass as mentioned above, must be rated as the reason for higher antioxidative capacities.

Results obtained within acetic acid analysis have to be analysed with caution as they seem very high. After consultation of the PFR laboratory this can be related to a faulty standard, used within the analysis. Production of acetic acid by spoilage organisms can be excluded due to the fast workflow at PFR and the prevention of oxygen contact. Still as all samples are affected, correlations should be seen as true effects.

No difference effect of different inoculation rates on acetic acid was detectable. A significant difference (P=0.009) could be seen within the different Superfood dosages though. SF2 lead to lower yields in acetic acid. Differences were as high as 0.127 g/l. Acetic acid is the main component of volatile acidity. Although other acids are known as volatile like formic, butyric and propionic, volatile acidity is usually only measured in wine as present as acetic acid (Jackson et al., 2008). Higher turbidity levels were previously correlated with lower yields of volatile acidity (Delfini & Costa, 1993; Nicolini et al., 2011). As the introduction of sterols and fatty acids is the main effect of turbidity on yeast, it is possible to connect acetic acid yield with sterol, UFA supplementation. Although no turbidity was adjusted within the inoculation trial, the application of SF2 may have had a similar effect by adding yeast cell wall components, derived by Yeast Hulls Nutrex 370 within Superfood. The synthesis of acetic acid was described by Delfini & Costa (1993) as an effect of the yeast trying to synthesize fatty acids in highly clarified media. This synthesis requires the production of Acetyl-CoA, a precursor for fatty acids, which cannot be utilized due to fast occurring lack of molecular oxygen in a ferment and will therefore hydrolyse into acetic acid. At lower doses (smaller than 300 mg/l), acetic acid can be a desirable addition to flavour and complexity of taste. It is especially important for the production of acetate esters. Above 300 mg/l acetic acid is known to taint other fragrances in wine and progresses to give wine a sour taste (Jackson et al., 2008)

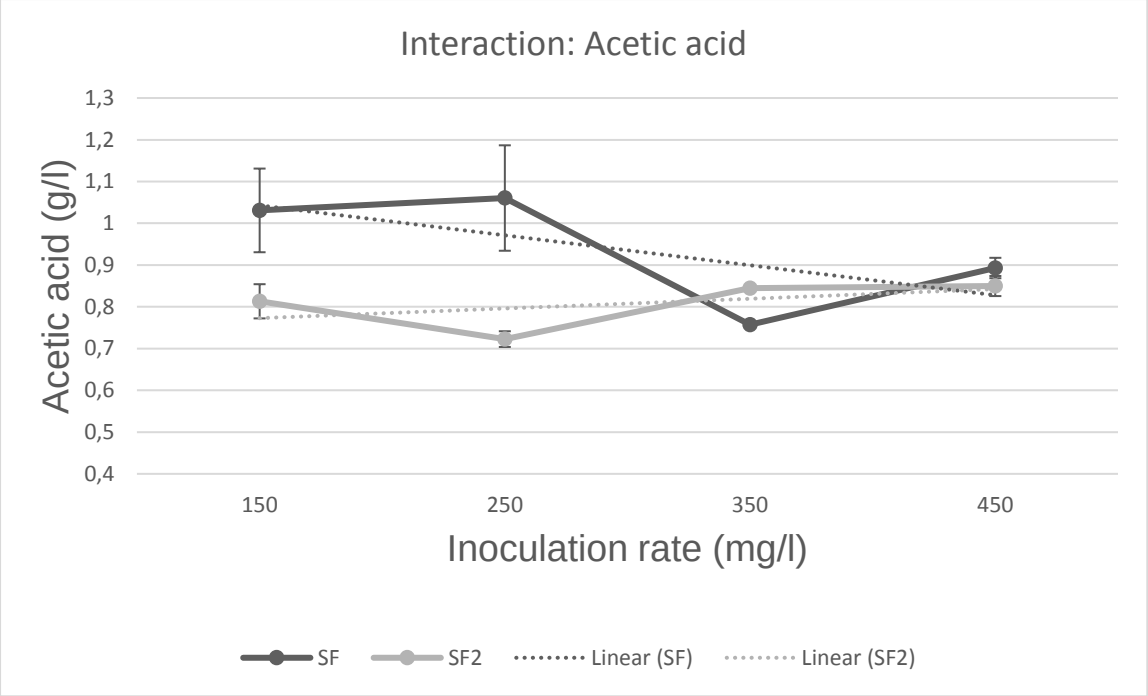

Figure 14 Interactions between the single and the double Superfood dosage as found for acetic acid (g/l) values within the Inoculation trial plus trendlines and error bars

Although significant difference has been detected between the SF and the SF2 dataset, a closer look into the interactions shows that the SF dataset showcases a negative trend whereas the SF2 dataset shows a slightly positive trend on higher inoculation rates. Further datapoints in both sets are quite inconsistent. The data obtained for 350 and 450 mg of yeast inoculations in addition are set very close which reveals multiple interpretation possibilities plus the need for further research. No clear conclusions should be drawn according to the acetic acid values measured during the inoculation trial.

Delfini & Costa (1993) found the production of succinic acid occurring in the same pattern as the production of acetic acid as the initiation of fatty acid synthesis and production of acetyl-CoA is activated by succinic acid. This correlation could not be shown within the inoculation trial as it already gets visible when comparing the trendlines in ***Figure 14 and 15***. Analysis of Succinic acid showed significant (P=<.001) differences in between the different amounts of yeast inoculated to the juices ***(Table 6)***. Differences were detected between the 150, 250 and 350 mg aswell as 150, 250 and 450 mg inoculation doses. No difference was detected between the addition of 350 and the addition of 450 mg of yeast. An overall trend of higher succinic acid levels at higher inoculation rates was visible with differences between the lowest and the highest inoculation dose of 0.414 g/l. No statistic effect of SF2 was detected. An interaction (P=<.001) between Superfood and the dose of yeast was found.

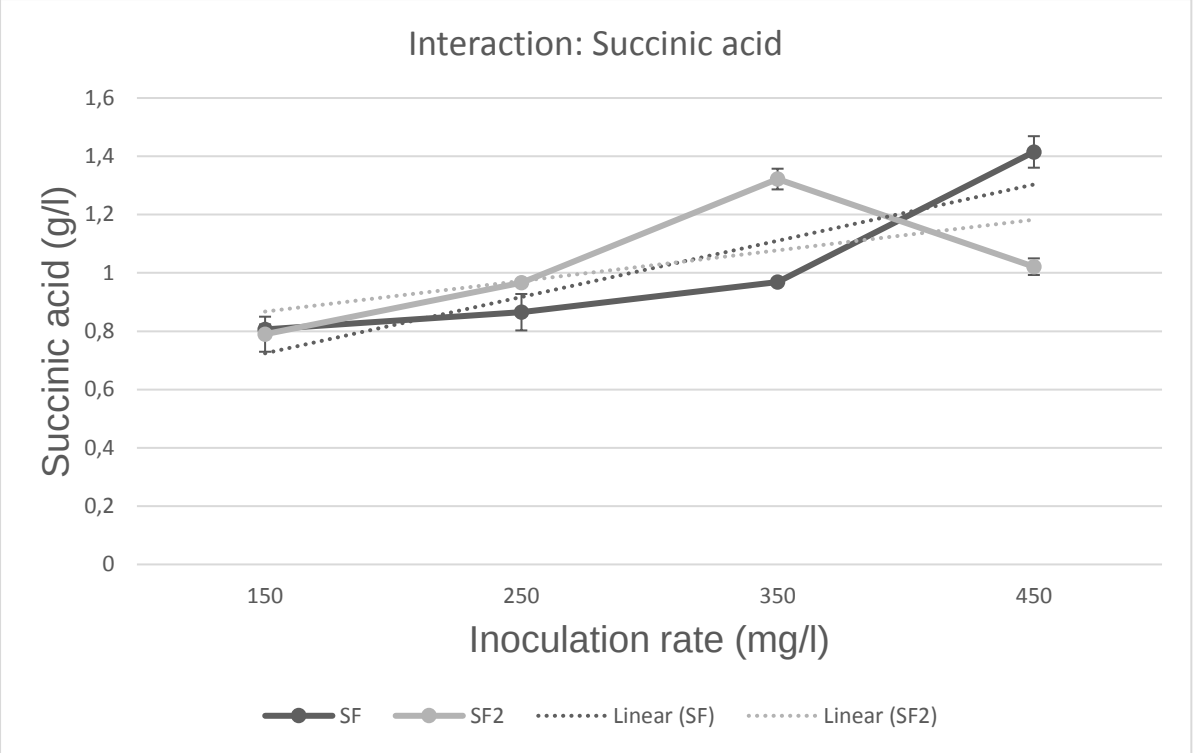

Figure 15 Interactions between the single and the double Superfood dosage as found for succininic acid (g/l) values within the Inoculation trial plus trendlines and error bars

Looking at the interactions found during the analysis of succinic acid, it must be mentioned that only SF dataset showcased a clear trend of higher succinic acid levels at higher inoculation doses. The SF2 dataset was more inconsistent as the 450 mg of yeast dose showed lower values than the 350 mg datapoint. Further, the 150 and 250 mg/l datapoints for both superfood additions were really close which makes overall declarations about succinic acid hard to rate within this trial. Succinic acid interactions follow a very similar pattern as interactions of malic acid, shown in ***Figure 11***. It must therefore be suspected that production of succinic acid underlies the same metabolic pathway as the conversion of malic acid at lower inoculation rates and longer fermentation time and that the production of succinic acid from malic acid may also be affected by the same enzymes, responsible for the MEF. As described above, the production of succinate is part of the anaerobic pathway of the MEF where malic acid it is transferred via fumarase to succinate, from where it is transferred to ethanol (Boles et al.,1998; Redzepovic et al., 2002, Saayman & Viljoen-Bloom, 2006).

Succinic acid plays an important role in the sensory profile of wine. It is known for its salty and bitter taste and takes part in ester formation like ethyl succinate or diethyl succinate in certain wines (Webb et al., 1964; Antonelli et al., 1999; Jackson, 2008). Succinic acid does not naturally occur in grapes of the *vitis* genome. It is produced by yeast of the genus *saccharomyces* during the fermentation as an intermediate of the citrate cycle and its derivates (de Klerk, 2010). It was determined, that succinic acid is the predominant non-volatile acid, formed during fermentation and it is the third most important acid in wines which have not

undergone malolactic fermentation (Thoukis et al., 1965). Amounts obtained normally reach from 0.2–1.7 g/l (Heerde & Radler, 1978). Highest yields of Succinic acid are produced by an oxidative pathway of the TCA cycle in the early phase fermentation till an alcoholic level of 4-5 % is reached, mainly due to the oxidation of glutamate and by a lower yielding reductive pathway in the later phases (Thoukis et al., 1965; Heerde & Radler, 1978; Arikawa et al., 1999). Yeast is also capable to transfer malic acid into succinic acid at rates between 0.8 and 1.5 g/l malic acid during fermentation (Heerde & Radler, 1978; Panda, 2011). Thoukis et al., (1965) have shown the correlation between higher amino acid content and higher succinic acid yield. This may be due to the direct effect of amino acids on yeast cell (Ribereau-Gayon et al., 2006b).

A significant effect (P=<.001) on yeast inoculation rate on reducing sugars was detected, between all inoculation rates finally making the highest yeast dosage showcasing the lowest amount of reducing sugars followed by all others. Yeast dosage and reducing sugars showed an inverse effect. The difference between the highest and the lowest yeast amount introduced to the ferment was 0.845 g/l. Further superfood had a significant effect (P=<.001) on reducing sugars. SF2 ferments showed lower amounts of reducing sugars, being 0.085 g/l lower than SF wines. Finally, an interaction (P=<.001) between superfood and yeast inoculation rate on reducing sugars was detected. Within the PFR protocol, reducing sugars are calculated as the sum of glucose and fructose, measured by enzymatic assay measurement. Glucose and fructose add to the reducing sugars but by the schoolbook, also yeast non fermentable pentoses are included in the reducing sugars. In a wine fermented to total dryness, only these non-fermentable, reducing sugars would be found. The PFR winemaking protocol advises to sulphur and chill wines fermented to 2 g/l of residual sugars, previously detected within a “Clinitest” flashtest. This causes small amounts of glucose and fructose still to be found in the wine unfermented. As ferment measurements normally are only carried out once a day and decisions about sulphuring wine is only possible to be made once a day, residual sugars may vary within the different wines and may cause differences within the residual sugar measurements, even if they do not describe any treatment effect.

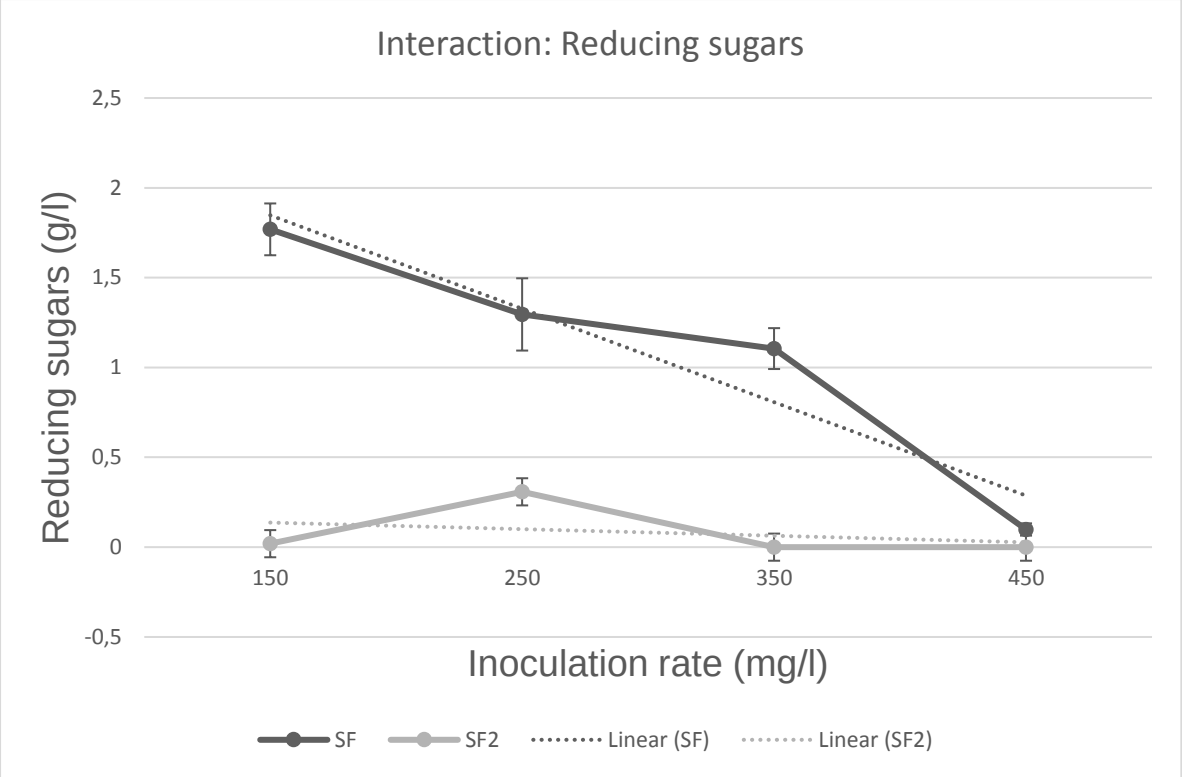

Figure 16 Interactions between the single and the double Superfood dosage as found for reducing sugar (g/l) values within the Inoculation trial plus trendlines and error bars

Interactions show that the main effect of reducing sugars must be described as been derived from the SF dataset. For SF2 overall differences were close to 0 and all datapoints close to 0 g/l. Therefore, a minor decline at higher inoculation doses was monitored. The datapoints at an inoculation level of 450 mg/l furthermore didn't show any statistical difference.

As reducing sugar analysis underlies the methodology of PFR, results obtained for the analysis of glucose and fructose should not be rated.

5.1.3 Thiol analysis:

Table 7 Thiol analysis

Analysis	150 mg/l Yeast dosage	250 mg/l Yeast dosage	350 mg/l Yeast dosage	450 mg/l Yeast dosage	P
3MH * (ng/l)	984	1021	1132	854	0.064
3MHA ** (ng/l)	**204.3 a**	**204.7 a**	**210.3 a**	**156.7 b**	**0.025**
4MMP *** (ng/l)	**154.0 a**	**141.7 a**	**158.7 a**	**103.7 b**	**0.034**
Sum of thiols	8.49	8.77	9.63	7.25	0.055
Ratio of thiols	0.16	0.15	0.14	0.14	0.079

Results of the Thiol analysis on wines obtained from the Inoculation trial. Means followed by different letters within a row are significantly different at the least significant difference level of 5 % (Fischers protected LSD)

* ***3MH = 3-Mercaptohexan-1-ol***

** ***3MHA = 3-Mercaptohexyl acetate***

*** ***4MMP = 4-Mercapto-4-methylpentan-2-one***

No statistical effect of inoculation rate on 3MH has been detected. Still, the 450mg/l trial showcased the lowest amount of 3MH.

A significant effect (P=0.025) of inoculation rate on the expression of 3MHA was detected. The 450 mg/l trial differed from all the other samples. The 450 mg/l sample showcased the smallest expression of 3MHA.

The expression of 4MMP was significantly (P=0.034) influenced by the amount of yeast inoculated. The 450 mg/l trial showed lower expression of the thiol than all other inoculation rate.

Although differences within the 3MHA and 4MMP analysis have been detected and a further trend within the analysis of 3MH, no overall difference in the total sum of thiols was visible. A trend of the 450 mg/l dose resulting in the lowest total amount of thiols detected in this trial.

Also the conversion rate of 3MH to 3MHA was not changed due to different inoculation levels, a trend towards much lower conversion at 450 mg/l was visible though.

These results indicate that inoculation rate has no positive effect on thiol expression and that bigger amounts of yeast can even have a negative effect on some thiols. An increase in inoculation rate for the meaning of higher thiol yields does therefore make no sense. **The** results confirm that for PFR's small scale winemaking protocol an inoculation rate of 250mg/L allows a wine to reach its full thiol forming potential if all other factors are favourable. As all juice was derived from one initial block, the question remains if juices with different composition might react different when inoculated with different doses of yeast.

As previously reported, the main differences seen in the fermentation curves occurred due to higher initial biomass, within the stationary phase of fermentation. Afterwards fermentation curves behaved equally. Within the SF2 dataset, differences in fermentation time were minor. All ferments finished fermentation after 9 days of fermentation, except of the 150 mg inoculation dose which took 10 days to ferment to dryness. Therefore, it must be suspected that enzyme activity during the first onset of fermentation is not strong enough to actually generate significant differences within the ferments and that further differences in fermentation time were not strong enough to trigger bigger differences. Further as both, the production of 4MMP aswell as the transformation of 3MH to 3MHA have been described as dependent on the yeast enzymes beta-lyase and alcoholacetyltransferase, it must be suspected that higher inoculation doses, when passing a threshold will end in lower enzyme activity. This may be due to quick depletion of amino acids in the medium certain factors required for fermentation as UFAs, Vitamins or trace elements which finally ends in weaker enzyme activity.

More detailed research should be carried out to clarify the phases during fermentation which are most important for thiol expression.

5.2 Turbidity trial:

5.2.1 Fermentation kinetics:

Table 8 Juice analysis as obtained during the turbidity trial

Analysis	Tuamarina Block	Gifford´s Creek Lane	Omaka Block
°Brix	20.9	20.1	20.7
Reducing sugars (g/l)	238.0	221.8	236.7
Glucose (g/l)	122.5	115.0	121.2
Fructose (g/l)	115.5	106.9	115.5
pH	3.01	3.12	3.01
TA (g/l)	8.8	9.7	8.8
Tartaric (g/l)	7.0	6.4	6.1
Malic (g/l)	4.0	7.0	3.5
YAN (mg/l)	141	251	88
Aas (mg/l)	100	201	64
NH_4 (mg/l)	41	50	23
Polyphenols (mg/l GAE) **	17	150	178
Folin Ciocalteu (mg/l GAE) **/***	4	7	5
A420/A320*	0.014	0.017	0.014

The table shows means as calculated from juice analysis of different origins.

* *A420/A320 is an oxidative index*

** *GAE = Gallic acid equivalents*

*** *Winescan derived data*

**** *NH_4 = Ammonia*

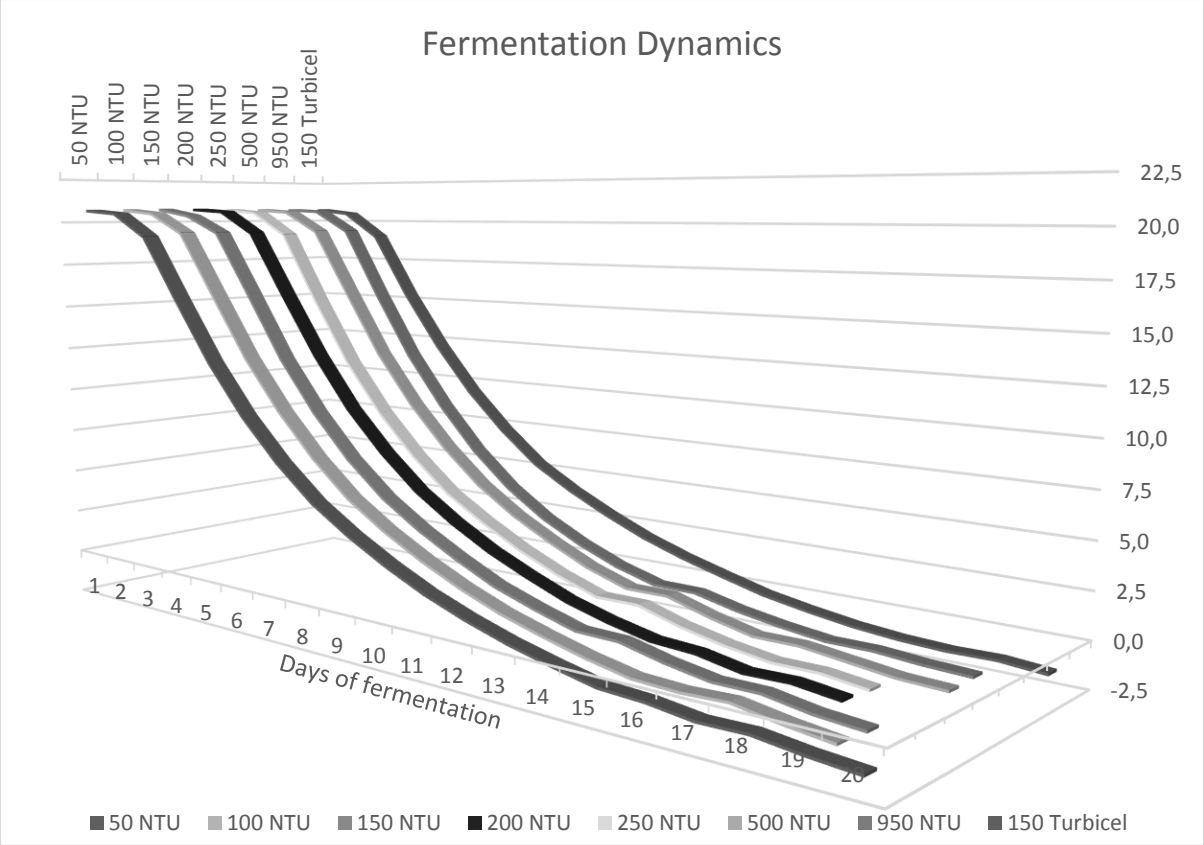

Figure 17. Mean fermentation dynamics as monitored during the turbidity trial. Values as shown on the vertical axis describe °Brix.

A significant treatment effect (P=0.006) on fermentation time was monitored ***(Table 9).*** Therefore, fermentation time was further used as a covariate, within statistical analysis.

Table 9 Fermentation times

Treatment	Tuamarina Block	Gifford`s Creek Lane	Omaka Block
50 NTU	17	15	20
100 NTU	16	15	19
150 NTU	16	13	19
200 NTU	16	14	**18**
250 NTU	16	**12**	**18**
500 NTU	**15**	**12**	19
950 NTU	16	**12**	19
150 NTU TC*	18	17	20

Shows different turbidity levels given in Nephelometric Turbidity Unit (NTU) as used during the turbidity trial and the day at which the fermentation dropped reducing sugars below 2 g/l.

**** TC = Turbicell, an inert Cellulose product used to increase juice turbisity***

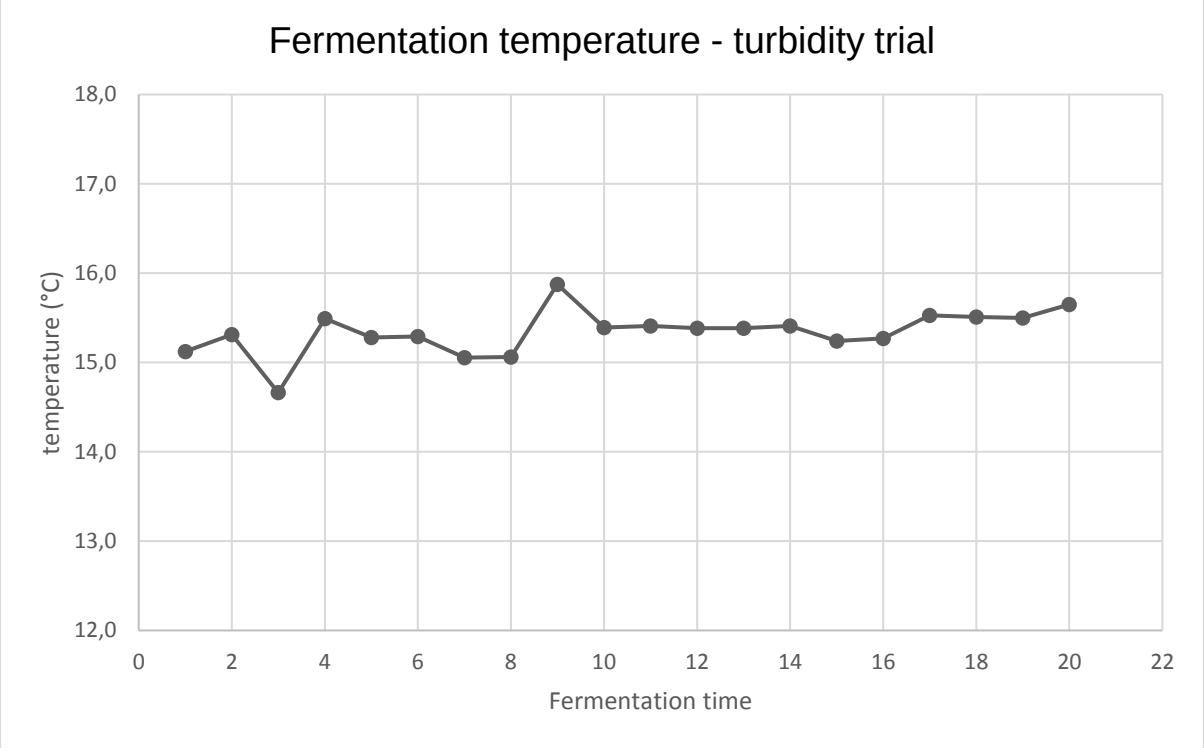

Figure 18. Fermentation temperatures as recorded during the turbidity trial.

All ferments fermented to total dryness. Ferments of the organic TB, finished their fermentation between 15 days (500 NTU) and 18 days (150 NTU TC). The ferments originating from GCL took between 12 days (250 NTU, 500 NTU, 950 NTU) and 17 days (150 NTU TC). Ferments of juice coming from OB finished between 18 days (200 NTU, 250 NTU) and 20 days (50 NTU, 150 NTU TC) of fermentation.

After yeast cell growth was monitored for the first 72 hours after inoculation. It was decided to stop further yeast cell counts. Reason for that decision was that solids started to separate into a foamy phase on top of the bottles and a fluffy phase on the bottom of the bottle. In between, a phase of fermenting juice was found. To extract a sample, as representative as possible, bottles were mixed gently before samples were taken, to ensure homogeneity of the ferment. Mixing of the bottles was done as it was seen that yeast cells attach to solids and therefore were not distributed similarly. Mixing of bottles was not only not sufficient to dissolve flakelike solids into the liquid but also lead to a CO_2 release that tend to floatate some solids to the top of the bottle immediately. Therefore, samples taken varied strongly and were not to be used to deliver representatively cell numbers. Similar behaviour of solids during fermentation was described previously (Casalta et al., 2009). These authors showed that the behaviour of solids can be divided into 3 phases, depending on the CO_2 production of the ferment. In a first phase, when yeast cells are in lag phase and CO_2 production is minor, solids would settle at the bottom of the fermenter. The second phase was described as the main part of the yeasts growth phase, where larger sized particles would break up and solids would dissolve into the ferment with the help of CO_2. In the third phase finally, during the stationary phase of fermentation, some of the dissolved solids would re-aggregate and form a deposit at the bottom of the tank. Casalta et al., (2009) concluded therefore that initial must turbidity cannot be considered consistent or representative during fermentation. After consultation of Farhana Pinu (The university of Auckland), it became clear, that other institutions make use of steady rotating fermenters, which ensure a more homogenous mixture of the ferments, together with higher repetitions of yeast cell counts to obtain representative yeast cell count numbers.

Table 10 Organic acid analysis

Analysis	Tuamarina Block	Gifford`s Creek Lane	Omaka Block
Tartaric acid (g/l)	7.0	6.4	6.1
Malic acid (g/l)	4.0	7.0	3.5
Ratio Malic acid / Tartaric acid (%)	35 %	51 %	34.7 %
Ascorbic acid (g/l)	0.18	0.15	0.12
Citric acid (g/l)	0.20	0.18	0.46

Mean organic acid content in juices of the three vineyards of the turbidity trial as obtained from organic acid analysis by High-performance liquid chromatography (HPLC) .

No statistical differences were seen, during the analysis of organic acids of the juices by HPLC ***(Table 10).*** GCL showed a very interesting acid profile though when compared to TB and OB. Malic acid values overcame the tartaric acid value by 0.609 g/l. This result stands out, as it is widely known that at maturity tartaric acid exceeds malic acid. Further it was shown that acids are highest at veraison and proceed to decrease from this point. The degradation of malic acid is stronger during the ripening period than the degradation of tartaric acid, which leads to tartaric acid being found at higher values at harvest (Liang et al., 2011). It is also known that high yields (27t/ha at GCL) can decrease maturation of grape berries and reduce acid degradation and overall grape quality (Jackson et al.,2008). If we compare the malic acid to titratable acidity ratio, it gets visible, that we can consider fruit from the GCL vineyard the least mature. Still, as results are not different within the statistic tests, more research has to be carried out to draw conclusions according to maturity.

Table 11 ***Table of means and statistical relevance of wines derived from the turbidity trial***

Analysis	50 NTU	100 NTU	150 NTU	200 NTU	250 NTU	500 NTU	950 NTU	150 NTU TC*****	Vineyard units stratum Covariate	Vineyard units stratum Treatment
A420/A320 *	**0.0280 cd**	**0.0284 d**	**0.0257 bcd**	**0.0236 b**	**0.0238 bc**	**0.0187 a**	**0.0167 a**	**0.0297 d**	0.093	**<.001**
Folin Ciocalteu ***	**370 c**	**349 bc**	**360 bc**	**348 bc**	**341 bc**	**323 b**	**269 a**	**335 bc**	**0.005**	**0.022**
Malic acid (g/l) Winescan	3.4	3.3	3.4	3.5	3.5	3.3	3.5	3.3	0.470	0.572
pH	**3.02 c**	**3.01 c**	**3.01 c**	**3.01 c**	**3.02 c**	**2.98 ab**	**2.97 a**	**3.00 bc**	**0.006**	0.754
Titratable acidity (g/l)	9.5	9.5	9.5	9.6	9.6	9.8	9.9	9.6	0.317	0.773
Reducing sugars (g/l)	1.0	1.1	0.8	0.7	0.5	0.7	0.6	0.8	0.322	0.970
Polyphenols (mg/l)**	177	177	173	172	175	170	170	172	0.182	0.093
Alcohol (%) corrected ****	**12.6 b**	**12.6 b**	**12.6 b**	**12.6 b**	**12.54 b**	**12.51 ab**	**12.46 a**	**12.54 b**	**0.015**	**0.033**

Means followed by different letters within a row are significantly different at the least significant difference level of 5 % (Fischers protected LSD)

* ***A420/A320 is an oxidative index***

** ***given in mg/l GAE = Gallic acid equivalents***

*** ***Folin Ciocalteu = Winescan derived data***

**** ***Alcohol, after calculative conversion of reducing Sugars***

***** ***TC = Turbicell, an inert cellulose product to adjust turbidity***

5.2.2 General results:

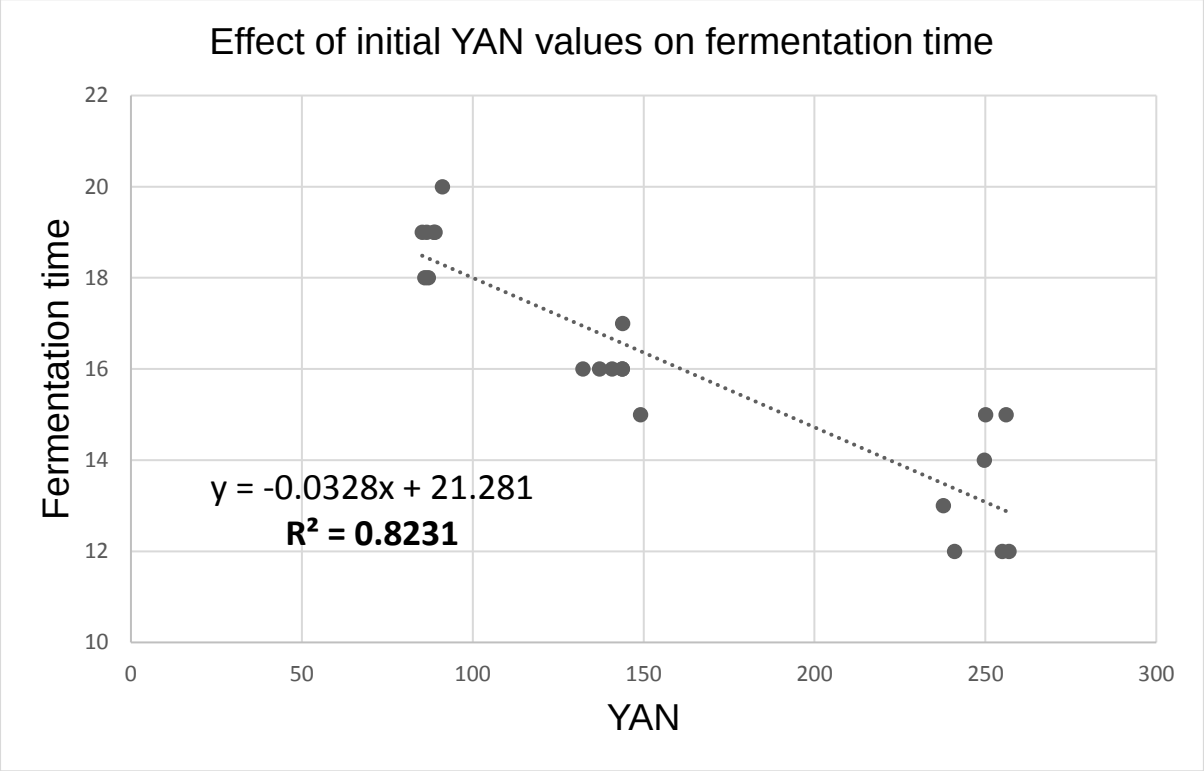

Figure 19. Shows correlation between initial YAN levels and fermentation time within the turbidity trial.

Figure 19 shows the correlation of initial YAN and fermentation time. It is visible, that original YAN levels at each vineyard site had a greater effect on fermentation time, than the main juice turbidity treatment within the TE and AS sample and that main effects of turbidity are visible only at the GCL vineyard.

YAN describes the addition of primary or alpha amino acids (excluding yeast non accessible proline), ammonium ions (NH_4) and small peptides and it is a common practise to correct YAN to ensure a non-sluggish fermentation. At PFR, all musts are therefore adjusted to 250mg of YAN before fermentation. It is known though, that a simple YAN correction by DAP may not be sufficient to ensure fermentation properties. Juices from low YAN vineyards are known to be depleted in other essential nutrients as well and may although nitrogen adjusted still produce some H_2S (Ugliano et al., 2007; Nowak et al., 2013). Therefore, the addition of complexer nutrients, which also supplement vitamins, lipids and minerals is recommended. At PFR, “Superfood” is therefore added to all juices as mentioned above. The reasons for differences in YAN values between vineyards can have multiple reasons as rootstock, variety, site, climate, soil, applied cultivation practises as fertilization (for, timing and rate) and soil management, canopy design-shading and temperature (Ough et al., 1968; Bell et al., 1979;

Huang & Ough, 1989; Bell & Robson 1999; Stines et al., 2000; Perez-Harvey & Witting, 2001). In terms of behaviour towards ripening, it was reported, that all major forms of berry nitrogen except of ammonia, reach a point, whether it is prior to, during or at the end of ripening, when a maximum concentration is achieved (Bell & Henschke, 2005). Only the pattern of evolution later in ripening that seems to be variable and is undoubtedly influenced by a combination of genetics, cultural practices and environmental conditions (Löhnertz & Schaller, 1992).

Table 12 Nitrogen composition

	Tuamarina Block	Giffords Creek Lane	Omaka Block
% Amino acids	71.2 %	80.2 %	73.3 %
% Ammonia (NH_4)	28.7 %	19.7 %	26.6 %

Initial nitrogen composition of juices derived from the three vineyards, used in the turbidity trial given in percent amino acids and percent ammonia.

It was shown that total amino acid content of juices increases from veraison, due to a transformation of ammonium. Therefore, ammonium reaches a peak after veraison and then depletes towards maturity (Kluba et al., 1977; O'Kennedy & Reid, 2008; Garde-Cerdan et al., 2009). The data shown in ***Table 12*** is interesting under this aspect, as GCL, although yielding the highest and showcasing the least degradation of malic acid has the lowest % of NH_4 at harvest. Yeast shows preference and different fermentative behaviour towards the composition of a nitrogen profile. Yeast prefers simple nitrogen sources and therefore NH_4 is known to be used immediately after inoculation, during the first 60 h of fermentation (O'Kennedy & Reid, 2008). During this time, the usage of amino acids is inhibited (Nowak et al., 2013). Crepin et al., (2012) were able to classify nitrogen compounds in 3 groups, according to their time of utilization during fermentation. The same patterns were confirmed within multiple *saccharomyces cerevisiae* strains and different amino acid concentrations and patterns. Prematurely consumed (Lysine), early consumed (Aspartic acid, Threonine, Glutamic acid, Leucine, Histidine, Methionine, Isoleucine, Serine, Glutamine, and Phenylalanine), and late consumed (NH_4, Valine, Arginine, Alanine, Tryptophan, and Tyrosine). Kemsawasd et al., (2015) were able to further classify amino acids as good and bad sources of nitrogen, which makes it obvious that all practises, changing the nitrogen profile of a juice, will also effect fermentation patterns and behaviour of *saccharomyces cerevisiae*. Research revealed concentration dependant different usage patterns of NH_4. Further the preference patterns of amino acid uptake were linked to different genes involved in the activity of different permeases, SPS and NCR (Crepin et al., 2012). The timing was seen as a crucial

factor for nitrogen additions. Yeast was monitored to only be able to take up amino acids during the early stages of fermentation, i.e. the first 20-30 g/l of sugar fermented and later during fermentation amino acid uptake is suppressed to the greatest extent. NH_4 uptake follows the same pattern but uptake rates cease much later during fermentation (O'Kennedy & Reid, 2008). Thibon et al., (2008) already suggested that the uptake of thiol precursors may be supressed by DAP induced NCR as their structural form is closely related to amino acids and proved this assumption. NCR didn't supress precursor uptake but volatile thiol release was suppressed by the Ure2p regulator, protein which supresses IRC7 transcription.
Additionally, NH_4 added during the exponential phase of yeast growth increases yeast cell numbers, nitrogen added at the stationary phase, didn't have an effect on yeast population but on fermentation dynamics (Bely et al., 1990). Although commercial products aim to supply yeast with more than just simple NH_4, these products normally cannot mimic natural amino acid profiles. The addition of amino acids is in general unusual. Yeast cell hulls, as found in Superfood though, are known to be capable to release a limited amount of amino acids (Munoz & Ingledew, 1990). But Yeast cell hulls as Nutrex 370 are mainly used to provide sterols for cell wall synthesis and to absorb toxic by-products like short-chain (C8-10) fatty acids and pesticide residues (Yeast Hulls Nutrex 370 datasheet). Yeast Extract T154 is a known provider of amino acids and minerals as required by the yeast (Yeast Extract T154 datasheet).

In general:
Superfood consists to 10.0 % of YAN
Superfood consists to 32.5 % of DAP
DAP consists to 21.0 % of YAN
Thus:
Superfood delivers 68.3% of its N in the form of DAP
Superfood delivers 31,7% of N sources other than DAP
PFR:
Adjusts YAN 50% by DAP and 50% by Superfood
-> PFR introduces 15,8% N sources other than DAP

Equation 2 Showcases a calculative estimation of Nitrogen (N) sources delivered by Superfood and delivered as supposed by the PFR winemaking protocol.

The amino acid fraction of the YAN as found in juices used within the turbidity trial ranged between 71.2 % and 80.2 %. Therefore, it is expectable, that the addition of Superfood cannot mimic the amino acid profile of grapes and yeast fermentation dynamics are affected more by the original, vineyard derived YAN amount, than by the primarily NH_4 adjusted YAN levels. Kemsawasd et al., (2015) already clearly demonstrated enhanced performances of

saccharomyces cerevisiae, supplied with complex nitrogen sources. This effects will be visible to a higher extent when vineyard derived YAN levels are low and bigger amounts of nutrients have to be adjusted. The thiol release supressing effect of DAP addition has been reported above (Subileau et al., 2008). DAP induced NCR was shown to repress thiol release of cysteinlyated precursors.

In the case of the TB and the OB, the highest turbidity did not correlate with the fastest fermentation time ***(Table 9).*** This may be due to the fermentation inhibitory effect of short chain fatty acids and esters, which can be formed by yeast during the alcoholic fermentation by catabolism of long chain fatty acids (Lafon-Lafourcade, 1984). Duan et al., (2015) described an increase of hexanoic and octanoic acid as a result of UFA addition. Although this research focused on the impact of aroma on these compounds, in higher doses these can have negative interactions with the yeast. Short chain fatty acids and esters are known to suppress hexose transport into the yeast cell (Bisson, 1999). This effect may be the reason for stuck and sluggish fermentation in some case. Here, a slight fermentation slowing effect may have occurred at the higher dosages of turbidity. Interesting is that this effect gets forced at the vineyards which showed lower initial NOPA values. Besides linolenic acid, UFA values are said not to change during maturity (Roufet et al., 1987). But nitrogen levels of a vineyard may correlate with the UFA composition and therefore affect fermentation kinetics in wine. Albertin et al., (2011) have shown that fermentation speed is mainly related to yeast cell mass, more than influx properties into the cell. The effect of nitrogen concentration in biomass and fermentation dynamics has further been highlighted by Gutiérrez et al., (2012). Although results obtained in the research, formerly named indicated that rather the nitrogen concentration than the nitrogen source is important for yeast cell growth, trials have only been carried out with different nitrogen sources as a sole source of nitrogen. Interactions of a mixed nitrogen source were not investigated. Therefore it can be suggested that a higher yeast cell mass production, triggered by high DAP additions in combination with high sterol and fatty acids obtained from juice turbidity as found in some OB and TB juices, may have led to an reduced uptake of amino acids and low assimilable nitrogen status during the stationary phase of fermentation, which left ferments with higher turbidity rates actually nitrogen depleted at the end of fermentation, unable to finish fermentation as fast as the lower turbidity trials.

No effect by the turbidity treatments on malic acid could statistically be proven within Winescan analysis ***(Table 11)***. Therefore, no organic acid analysis on the wines was carried out in this trial. Further, no effect was seen in titratable acidity. Still, a statistic effect on pH values was detected within the single vineyards, caused by the covariate fermentation time. Differences were detected between 950 NTU and 250 NTU, 200 NTU, 150 NTU, 100 NTU, 50 NTU, 150 NTU TC. Further differences existed between 500 NTU and 250, 200, 150, 100. 50 NTU.

Differences, although shown as statistically relevant, did not exceed 0.051 values, which showcases a minor effect on the need of winemaking decisions. pH value has to be seen in connection with titratable acidity measurements. No statistic difference in titratable acidity was detected in the trial. But still a trend towards higher TA at shorter fermentation times was seen. Measurements of 200 NTU showing higher values than measurements of 250 NTU can be seen as an artefact. Differences between the lowest and the highest turbidity did not exceed 0.357 g/l. As no differences in malic acid were detected, significance as found for titratable acidity must have been caused by other organic acids, which were not further analysed here, most probably tartaric acid. It would be expectable, that higher yeast cell mass, as expected at higher turbidities releases a bigger rate of mannoproteins which prevent tartrate crystallisation already during fermentation and therefore remain higher titratable acidity in the wines (Laubers et al., 1987; Boissou et al., 2007).

Further pH correlates with the amount of potassium (K) found in a juice. Potassium is mainly accumulated in the berry skins (Jackson et al.,2008). As mentioned above, turbidity mainly consists of skin fragments and cellular debris, from grapepulp (Ribereau-Gayon P. et al., 2006b). Therefore, higher potassium levels have to be expected from higher turbidity levels, which could increase pH value. An anti-thesis to this is, that suspended solids have an absorptive effect on potassium and natrium ions and therefore are able to prevent an increase of pH as monitored during the turbidity trial (Joshi et al., 2013). No analysis of cations has been conducted though, to verify this statement.

No statistic difference has been detected caused by the treatment or by the fermentation time in reducing sugars. Values ranged between 0.453 g/l and 1.104 g/l. As described within the inoculation trial, reducing sugars as measured within the PFR protocol are rather affected by the timing of sulphur addition and the stop of fermentation rather than by an effect of the actual treatment within the trials.

No statistic differences in polyphenols have been detected ***(Table 11).*** Not of the main treatment or of fermentation time as a covariate. As solids, used in this trial were suspected to consist mainly of cell debris, including berry skin fragments, it would have been expectable that an increase of polyphenols could have been measured. This was not the case as no significant differences within the treatments has been monitored. These results emphasize the results of Singleton et al., (1975) who were also not able to find an increase in polyphenols in wines, fermented turbid. These results exclude the hypothesis drawn by Joshy et al., (2013)

who mentioned a higher possibility of phenol oxidation and a coupled thiol oxidation via quinones.

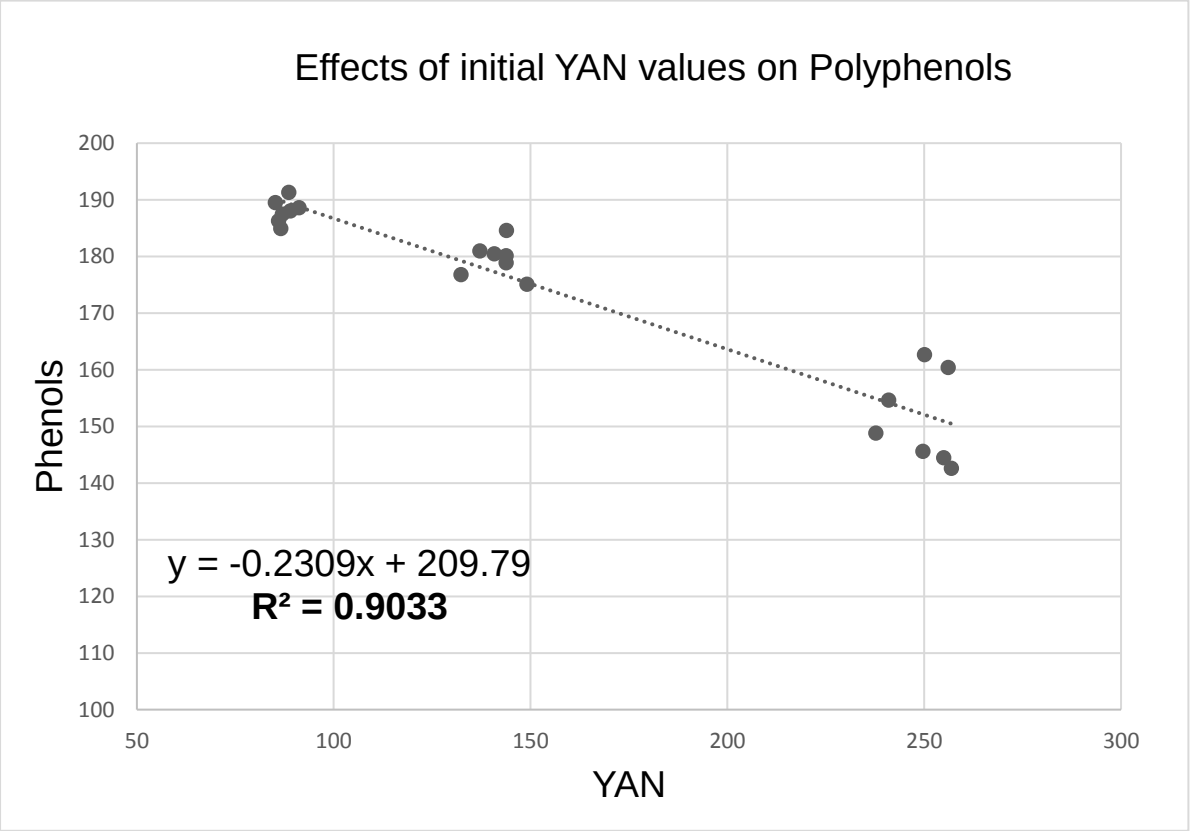

Figure 20. Shows correlations between initial YAN levels and Polyphenols within the turbidity trial.

Although no statistic effect of the treatment on polyphenols could be detected, a correlation between YAN values and polyphenols was monitored. ***Figure 20*** shows these correlations between initial YAN values and polyphenols. Correlation between nitrogen status of a vineyard and polyphenols have been researched previously on Tempranillo grapes by (Delgado et al., 2004), who found out that vines without nitrogen fertilisation accumulated higher doses of polyphenols in the skin than vines with fertilization. Equal effects were detected by Keller & Hrazdina, (1998) at the variety Cabernet Sauvignon, where a fertilization of nitrogen had a delaying effect of polyphenol accumulation, although differences became less at maturity. The decrease of polyphenols in vineyards with higher nitrogen status has been linked to an increase in vigour (Downey et al., 2005). As no soil analysis have been carried out within this trial, we can only take visual observations as an indicator for the nutritional status of the vineyards. Visual observations have shown though that the OB vineyard was the lowest in vigour, followed closely by TB. GCL showed the highest vigour. This pattern equals for the

initial YAN values in the juices and the polyphenols, detected in the wines. Research by Kamiloğlu, (2011) on 'Horoz Karası' have shown a negative effect of high yields on total polyphenols. Equal results were monitored by Prajitna et al., (2007) on the variety Chambourcin, where lower yields lead to a higher final polyphenol content. As YAN is directly affected by crop load, YAN as an indicator of nitrogen status of a vineyard have to be evaluated with care. Yield furthermore is influenced by rootstock, vine clone, soil properties, nutrients, weather and human practises. The relatively low differences in polyphenol content, although bigger differences within YAN values between the TE and OB vineyard have been detected, have to be rated as an interaction of higher crop yield and low vigour, as observed at the OB vineyard.

A significant effect of both, juice turbidity (P=0.033) and fermentation time (P=0.015) on ethanol yield was detected. The turbidity of 950 NTU yielded in less alcohol than 50 NTU, 100 NTU, 150 NTU, 200 NTU, 250 NTU, 150 NTU TC. No difference has been detected between 50 NTU, 100 NTU, 150 NTU, 200 NTU, 250 NTU, 500 NTU and 150 NTU TC. Further no difference could be found between 950 NTU and 500 NTU ***(Table11)***. Although ethanol analysis showed statistical difference of mainly the 950 NTU treatment, maximum difference detected didn't exceed 0.11 %. This results excludes the relevance of the effect of turbidity on daily winemaking techniques. When taking a closer look at the single replicates, the 950 NTU treatment always had the lowest ethanol yields. Actual alcohol levels between treatments were very low and again differences between the vineyards were the main cause for derivation. As no differences within malic acid were detected, a difference in % alcohol cannot be described by the action of MEF. More detailed research about the interaction between organic acids and turbidity has to be carried out to understand effects on ethanol yield.

Significant correlations (P=<.001) were detected between turbidity and A420/A320 measurements in Sauvignon blanc wines, obtained in this trail. Values ranged between 0.0175 and 0.02795, spanning a window of 0.01045 units. 950 NTU were shown here to have a lower value than 250 NTU,150 NTU, 100 NTU, 50 NTU and 150 NTU TC. 500NTU aswell showed a lower value than 100 NTU, 50 NTU and 150 NTU TC. No differences were detected between 50 NTU, 100 NTU and 200 NTU aswell as between 150 NTU, 200 NTU and 250 NTU. Allen at al., (2011) noted in research focusing about thiol expression in relation to oxidation, that wines with A420/A320 values below 0.03 units were the least oxidized, leading to 3MH concentrations >1000 ng/l. Further A420/A320 values >0.07 produced wines with a 3MH content <750 ng/l. Our overall results and especially the outcome of thiols analysed, as shown in table 13 showcase the effectivity of the PFR winemaking protocol to avoid oxidation and to allow the highest thiol expression. Reason for low oxidation is surely also handpicking of fruit, which prevents the action of oxygen till the actual processing steps at the winery. Further it

was shown, that oxidation levels did correlate with NTU levels, not with fermentation time as even wines gained from the TB and the OB vineyard showed least oxidation values at 950 NTU, although these turbidity levels did not ferment the fastest. This implements that a longer fermentation time does not affect oxidation levels as wines are fully protected by 1.) Dissolved CO_2, 2.) Oxygen consuming yeast and 3.) the Plant and Food protocol, involving regular use of argon gas to protect the wines is efficient, although slower ferments will have more regularly contact to air exposure while °Brix measurements. So what may be the reason for that? Besides sulphur, polyphenols are known to have a protective effect against oxidation. But no statistic difference between polyphenol levels was shown in this research. As suggested before, higher solids will influence, sterol and fatty acid levels in juice. These sterols and fatty acids are a limiting factor for a yeast population, which implements that juices fermented at higher turbidity rates, host higher yeast cell numbers (Burglass A.J., 2010). Yeast itself is known for its oxygen scavenging effects, as cells of *saccharomyces cerevisiae* use oxygen as a nutrient for their own sterol synthesis. It is therefore expectable, that a higher yeast cell biomass has higher scavenging capacities. This thesis confirms results obtained from the inoculation trial, where higher yeast inoculation rates were suggested to end in slightly higher yeast cell numbers and to be able to start fermentation earlier after inoculation.

5.2.3 Thiol analysis:

Table 13 Thiol analysis (turbidity trial)

Analysis	50 NTU	100 NTU	150 NTU	200 NTU	250 NTU	500 NTU	950 NTU	150 NTU TC*	Vinyard units stratum Covariate	Vinyard units stratum Treatment
3MH ** (ng/l)	**2359 a**	**2278 a**	**2297 a**	**2649 a**	**2433 a**	**4163 b**	**6439 c**	**2943 a**	**0.022**	**<.001**
3MHA *** (ng/l)	628	644	799	843	905	1166	1232	591	**<.001**	0.141
4MMP **** (ng/l)	94.6	90.8	76.6	75.6	59.7	66.0	88.1	120.6	**0.011**	0.330
Sum of thiols	**22.90 a**	**21.21 a**	**21.06 a**	**23.93 a**	**21.49 a**	**35.86 b**	**53.78 c**	**28.80 ab**	**0.007**	**<.001**
Ratio of thiols	**0.25 d**	**0.23 cd**	**0.23 cd**	**0.208 cd**	**0.207 c**	**0.17 b**	**0.12 a**	**0.23 cd**	0.490	**<.001**

Results of analysis of thiols from the Turbidity trial including a sum of thiols aswell as a conversion rate from 3MH to 3MHA.
Means followed by different letters within a row are significantly different at the least significant difference level of 5 % (Fischers protected LSD)

* *TC = Turbicell, an inert cellulose product, used to adjust turbidity*

** *3MH = 3-Mercaptohexan-1-ol*

*** *3MHA = 3-Mercaptohexyl acetate*

**** *4MMP = 4-Mercapto-4-methylpentan-2-one*

A significant (P=<.001) effect of turbidity on 3MH levels was detected. Both the 500 and 950 NTU showed higher expression of 3MH than all other trials. The 500 NTU level differed from the 950 NTU level, showing lower values of 3MH. No statistical difference was detected between 50, 100, 150, 200, 250 NTU and 150NTU TC. It was also shown that the covariate, fermentation time had a significant effect (P=0.022) on 3MH as longer fermentation times tended to showcase lower levels of 3MH. The 950 NTU treatment showed values up to 2.72 times higher in 3MH than other values. The 500 NTU treatment showed values up to 1.76 times higher than other treatments. Although wines obtained in this research were only produced for analytical analysis, which did not involve sensory or analysis of H_2S, It is very likely that samples with high turbidity as found in the 500 and the 950 NTU set, formed an increased amount of H_2S as it was formerly reported in juices fermented at high turbidity (Karagiannis & Lanaridis, 2002). H_2S is known to play an important role in the formation of 3MH under influence of (E)-2- hexenal as mentioned above (Schneider et al., 2006). There was not a similar effect seen on the production of 4MMP, therefore this thesis suggests that only 3MH formation is affected under normal fermentation conditions by H_2S. Similar results have been obtained by Pernod Ricard and an application for a method to modulate wine aroma has been recorded as a recently published patent (Thomson Innovation Patent Export, 2016-06-20 16:26:21 -0500). The patent describes a method which includes the introduction of H_2S enriched gas, obtained from a fermentation through a gas sparger to a ferment, starting 2 days before inoculation and ending within the middle of alcoholic fermentation. The introduced gas is described in the patent to show a H_2S content of 10ppm. Wines obtained from ferments threated with H_2S gas showed values 1.5 fold higher than unthreatened wines. Ensuring sparging of H_2S containing gas is stopped at the middle of fermentation makes sure no S-containing off flavours remain in the wine (carbon disulphide, dimethylsulphide, dimethyldisulphide). Although no analysis of such components has been carried out in this trial. It is suggested that S-containing off flavours have formed ion the samples fermented at higher turbidity and remained in the wines after fermentation, as previously monitored (Karagiannis & Lanaridis, 2002). As wines with lower turbidity generally fermented slower and were less likely to produce H_2S, suggests that fermentation time in this case showcased significance.

Significant differences (P=<.001) caused by the covariate fermentation time have been monitored for 3MHA ***(Table 13)***. The effect of fermentation time on 3MHA expression was not strong enough to cause significant difference between the treatments. As reported previously, the formation of 3MHA relies not on a direct precursor, but on a transformation of 3MH to 3MHA by the yeast`s alcohol acetyltransferase enzyme activity (Swiegers et al.,2005). Although no direct significance of the treatment was shown during this trial, there was a clear

trend towards a higher 3MHA expression at higher turbidities within all three vineyards. Especially in OB, which has been described previously as strong in vigour and high in nitrogen, an increase of 3MHA at higher turbidities was seen.

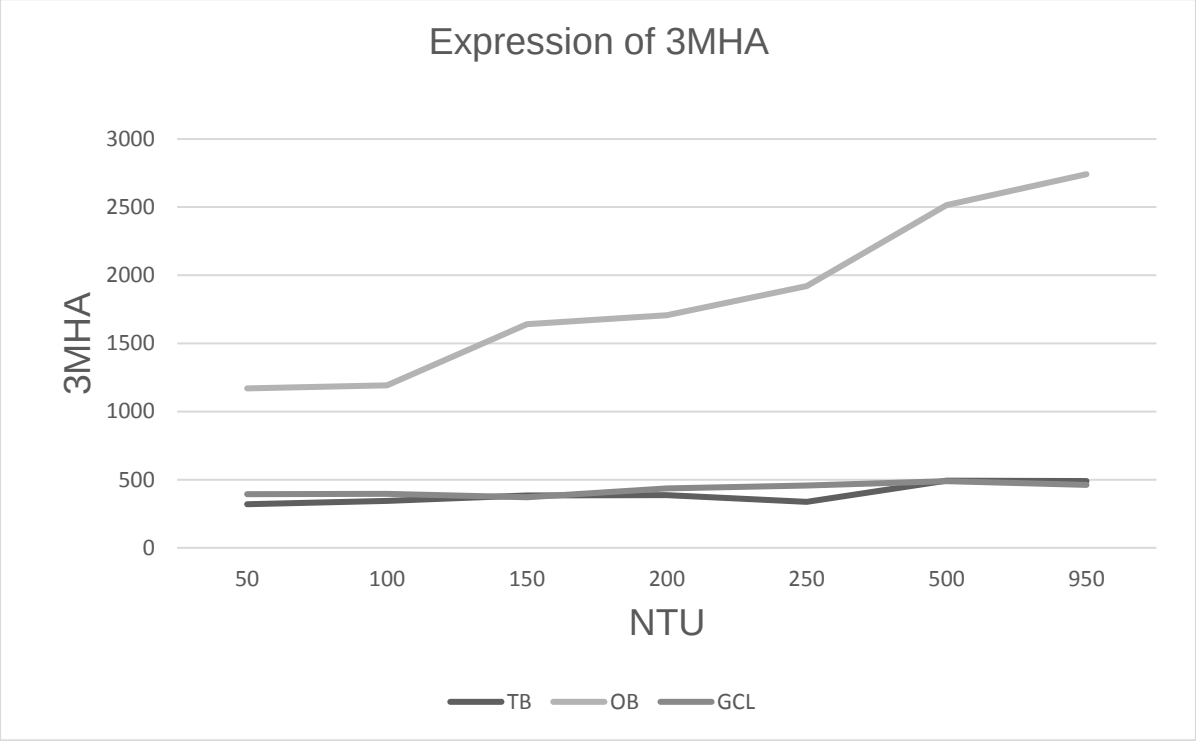

Figure 21 Shows the different expressions of 3MHA between the vineyards of the turbidity trial.

Although not significant, OB showed a 2.3-fold higher expression of 3MHA at the 950 NTU level when compared at the 50 NTU level. It should also be mentioned that both other vineyards, the TB aswell as the GCL had the highest expression of 3MHA at the 500 NTU turbidity level. Although a positive effect of UFAs on the expression of volatile aromas was reported by Duan et al., (2015), The thesis of a linear increase of alcohol acetyl transferase activity with higher turbidity, caused by the concentration of UFAs in the juice to be excluded. Furthermore, data obtained during this research showed a rather low correlation of 3MH to 3MHA ($R^2 = 0.4949$). This was also underlined when conversion rates of 3MH to 3MHA were reviewed. The conversion ratio of 3MH to 3MHA was significant affected by the turbidity treatments ***(Table 13)***. The 50 NTU treatment differed from the 250. 500 and 950 NTU treatment. The 250 NTU treatment differed from the 500 and the 900 NTU treatment. The 500 and 950 NTU treatments varied from all others, but also from each other. No differences were discovered between the 50, 100, 150, 200, 250 and 150 NTU TC treatment. A higher conversion rate was seen at lower NTUs, which has to be related to the expression of 3MH which was the highest at high turbidities. Therefore, the conversion rate as calculated cannot be seen as affected by 3MH concentration in the ferment but mostly on enzymatic expression

of alcohol acetyl transferase. A stronger correlation between initial YAN values and the expression of 3MHA was found though.

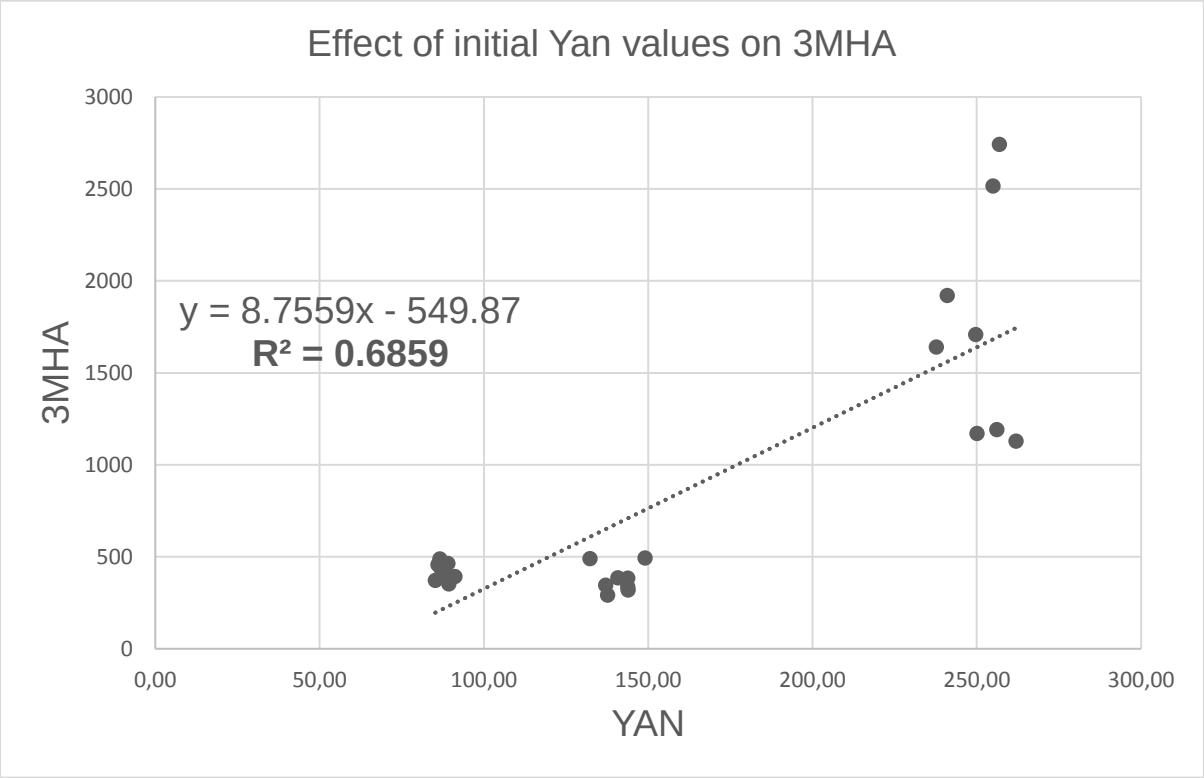

Figure 22 . Shows correlations between initial YAN levels and 3MHA within the turbidity trial.

These results indicate that the genetic response, leading to a transcription of alcohol acetyl transferase may be dependent on the initial amino acid concentration in the juice. Further research should be carried out to clarify this thesis. Although YAN values in all juices have been adjusted previously to fermentation, the concentration of amino acids was the highest in GCL, as reported previously. As ***Figure 22*** demonstrates, it is very likely that juices, high in AAs tend to convert bigger amounts of 3MH to 3MHA when fermented at higher turbidities. This effect seems to response in an exponential way once a certain amino acid threshold has been passed.

The expression of 4MMP was significantly affected (P=0.011) by fermentation time ***(Table 13).*** The effect of fermentation time on 4MMP expression was not strong enough though to cause significant difference between the treatments. As E-2-hexenal was reported to be a precursor to 3MH, mesityl oxide was suspected to be a precursor to 4MMP who would be accessible for yeast after interaction with H_2S. (Harsch et al., 2013). Difference in the pathway leading to either one of this compounds was supposed to be a sulphur addition on the double bond for 4MMP and a reduction of the carbonyl group in the case of 3MH. To my best knowledge the conversion of mesityl oxide to the precursors of 4MMP is a theoretical scenario as mesityl

oxide has not been found in grape juice derived from *vitis vinifera* so far. This fact does make a minor effect of H_2S applied to grape juice on the expression of 4MMP obvious.

The sum of thiols was significant affected (P=<.001) by the different turbidity levels ***(Table 13)***. The 950 NTU trial varied from all others. The 500 NTU trial differed from all others but the 150 NTU TC trial. No Difference has been detected between 50, 100, 150, 200, 250 and 150 NTU TC.

6.0 Conclusion:

Higher inoculation rates mainly affected fermentation time. In the SF dataset, fermentation time differed by 2 days within the different inoculation levels, in the SF2 dataset by 1 day. Fermentation kinetics showed, that most changes occurred during the lag phase, only minor effects could be seen during the stationary phase of fermentation. As the initial juices were only derived from one juice lot, results were very close within the replicates with low standard errors in average. Therefore, many statistical differences could be detected during analysis of the inoculation trial. Although inconsistent, inoculation rates, aswell as Superfood additions had an effect on the acidity profile of the ferments. Inoculation with higher doses of yeast lead to a decrease in pH value, but only at SF2. The differences between the lowest and the highest amount of yeast did not exceed a difference of 0.06. As pH is related to titratable acidity, it has to be mentioned, that differences in titratable acidity during the inoculation trial were affected by interactions which make a rating approach towards this result impossible. Therefore, it is questionable if this result implements any practical adaption possibilities. Although a trend towards higher levels of malic acid at higher inoculation rates was monitored, also here, interactions make ratings impossible, as a continuous ascend in malic acid from 150 to 450 mg of yeast per litre was only to be found in the SF dataset. Tartaric acid again underlies the strong effect of interactions and results are not continuously ascending. Still a trend of higher acidity at higher inoculation rates may be described within both datasets. The higher expression of succinic acid at higher inoculation doses must be seen mainly due to effects generated by the single SF addition, the behaviour of the SF2 dataset was rather inconsistent. Effects on succinic acid underlie strong interactions. Changes in alcohol content, although significant were only found in the SF2 dataset after interactions were detected. In this dataset difference between the 150 and 450 mg inoculated ferment were as high as 0.46 %.

A correlation may be drawn between the effect monitored at malic acid aswell as pH within the SF2 dataset, which suggest the use of higher doses of yeast in ferments with higher nitrogen supply can suppress MEF and therefore lead to wines with a lower alcohol content.

Additionally, it was seen, that higher yeast inoculations are able to preserve ascorbic acid in a ferment. Although results were not continuous, significant differences in the state of oxidation, described within the A420/A320 ratio were found within the different levels of inoculation, aswell as the different Superfood additions. Higher doses of yeast inoculated tend to show a lower A420/A320 ratio. Further, SF2 had a lowering effect on the A420/A320 ratio. Both, ascorbic acid values and the A420/A320 ratio implement that higher yeast inoculation doses can be used to reduce effects of oxidation during fermentation.

Although no changes in polyphenol content during fermentation was monitored, the Folin Ciocalteu index was affected by both, inoculation rate aswell as Superfood dose. Lower values were obtained at higher doses of inoculation, although no difference was seen between inoculation of 350 and 450 mg of yeast per litre. Further SF2 lead to a lower Folin Ciocalteu index. The Folin Ciocalteu Index was influenced by the practises applied at PFR. Therefore, no conclusions can be drawn according to this dataset.

Thiol analysis revealed differences to occur only for 4MMP and 3MHA, where levels obtained at 450 mg yeast inoculations were lower as all others. Further, it should be mentioned that there also was a trend towards the 450mg trial showcasing the lowest amount of 3MH. This trend was also found in the sum of thiols. This result is unexpected as lower thiols could be expected due to higher oxidation as described during A420/A320 analysis and especially due to the effect of H_2O_2, derived from the oxidative breakdown of Ascorbic acid. As none of these suggestions happened and the 450 mg/l inoculation showed the lowest expression more research has to be carried out to clarify this effect.

Within the turbidity trial, juices derived from 3 vineyards of the Marlborough region were used. This procedure caused less but strong significant results as the vineyards differed in their nutritional supply, yield and farming practises applied. The most interesting findings have to be mentioned to be the strong effect of the vineyards nitrogen supply. The effect of high initial YAN values had influence on thiols, stronger than expected and overcame the actual treatment effect. This behaviour was also monitored for polyphenols and fermentation time and was affected by crop yields or erased by adjustment of juice YAN as performed at PFR.

Although fermentation time was mainly influenced by the vineyards initial YAN, an effect of turbidity was monitored. Not for all vineyards the highest turbidity correlated with the fastest fermentation time. It seems that the composition of turbidity changes according to the nitrogen status of a vineyard and shows therefore different optimum rates and effects between the vineyards.

As monitored during the inoculation trial, turbidity, affecting fermentation time had effects on the pH value. It was seen that wines, fermented with higher turbidities tend to showcase lower pH values. At the same time these ferments yielded slightly reduced alcohol levels. As no changes in malic acid were detected within Winescan analysis, no analysis of organic acids was carried out. Changes in pH and titratable acidity cannot be described by the effect of MEF and more detailed research has to be carried out to understand the interaction between organic acids and turbidity.

During this trial a significant effect of turbidity A420/A320 values was detected. Wines derived from ferments fermented at 500 and 950 NTU showcased much lower values.

During the turbidity trial, thiol expression was increased significantly in ferments fermented with 500 and 950 NTU. These differences were mainly caused by a 1.76 respectively up to 2.72-fold increased expression of 3MH. Therefore, also significant changes within the analysis of 3MHA were seen, which were described as an effect of UFAs found in turbidity. Further, correlations were described between the initial, vineyard derived YAN values and conversion of 3MH to 3MHA. Although a trend towards higher 3MHA expression at 500 and 950 NTU was seen, no statistical treatment effect was found within analysis of 3MHA.

No effect of turbidity on 4MMP was detected, although a significant effect of fermentation time was monitored.

It has to be concluded, that a fermentation at high turbidities should not be rated as a sole method for an increase in 3MH. Ferments at higher turbidity have to be expected to form bigger amounts of H_2S and therefore also form more stable sulphur compounds which will create undesirable off flavours in the final wine. More practical applications have to be considered to be an introduction of gaseous H_2S to a ferment to trigger 3MH formation.

Further research should also be done on ferments with previously increased amounts of green leave volatiles.

7.0 Recommendations for PFR:

Besides to study the effect of turbidity on aroma compounds, aim of this work was to improve the small scale winemaking protocol for Plant and Food research and decrease the occurrence of stuck or sluggish fermentations as monitored in years before.

Due to the adjustment of turbidity, stuck and sluggish effects were prevented during the 2016 ferments by 100 %. Problems of turbidity adjustment can be described as below:

1.) Lack of accuracy of the “Hach-2100Q is” turbidity meter
2.) Slow workspeed of the “Hach-2100Q is” turbidity meter
3.) Differences in the racking technique of different people and even different racked juices, processed by the same person.
4.) Differences in settling and initial NTU.
5.) Time consuming multistep adjustment to reach accurate NTUs
6.) Time consuming temperature adjustment to measure NTUs

To improve the workflow within daily winery operations, the following options should be suggested.

I don’t recommend the acquisition of small scale juice clarification technics as floatation, yeast press or centrifuge as this equipment is too costly and same effects can be achieved by the following.

The introduction of gelatine into the PFR clarification protocol will further ensure clarification processes and will further compact solids during settling. Therefore juice yields can be increased during racking and the need to rack turbid juices in cases of low press yields can be further decreased.

1.) Purchasing of juice lees, produced by a commercial winery of the Marlborough region. These lees could be racked with a focus on fluffy lees within a multistep racking process. Afterwards the obtained lees could be analysed, a fixed dose for 0.7l and 18l fermenters could be estimated in a previous trial. Finally, lees could be cut into the desired doses, frozen and finally added as needed.
   Cons of this method would be that juice lees would have to be frozen very early in the season or stored for a whole year to be available at the beginning of the next season. Further the same lees would be used for every juice, possibly interacting with treatment

effects. Finally, chemical changes of must lees during freeze storage must be further investigated.

2.) Results of this trial revealed that addition of Turbicel alone, does not have an effect on the physical benefits of must turbidity, as it doesn't supply yeast with sterols or fatty acids. Furthermore, yeast nutrition at PFR is provided by a mixture of Superfood and 30 % DAP solution, which provides yeast cells mainly with easy accessible ammonia, which is used during the first days of fermentation, leaving yeast depleted at the end of fermentation as amino acid metabolism is inhibit and yeast mat be left depleted during the end of fermentation.

I recommend a change in yeast nutrition. To move away from superfood, towards a more complex medium, which doesn't supply yeast with simple ammonium sources but with amino acids, small peptides and sterols. A various range of organic media is available on the market, delivering only organic nitrogen, promising long term supplementation during fermentation.

The Following products caught my attention.

SIHA-Proferm Bio: Yeast cell wall preparation, Provides sterols, lipids and vitamins

ERBSLOEH-VitaFerm Bio: Yeast cell wall preparation, provides sterols, lipids and amino acids

UVAFERM Fermaid O: Yeast cell wall preparation providing amino acids and peptides

My idea was to introduce a multistep supply system, which would begin with the addition of Yeast cell walls and Turbicell before inoculation and a later adjustment of YAN by DAP after fermentation of the first 20-30g of sugar. This would enable the yeast to have sufficient access to sterols and fatty acids to build up biomass. Turbicell will provide the physical effect of turbidity, increasing the juices inner surface, needed for CO_2 release. The delayed addition of ammonia will force the yeast to take up higher quantities of amino acids and will therefore ensure a maximum uptake of amino sources. Benefits of this system would be that must lees after racking are supposed to be very compact and can be discarded completely. Addition of survival factors and adjustment can be done in the most precise way.

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9.0 Attachments:

Effect of juice turbidity on fermentation rate and wine composition of Sauvignon blanc wine

Background:

The development of robust small scale winemaking protocols is essential for reliable, repeatable wine research. The turbidity of juice during fermentation can influence the rate of fermentation and the potential flavour and aroma compounds (particularly varietal thiols) in Sauvignon blanc wine. To achieve consistent fermentation rates and reduce variation between replicate ferments it is important to understand the impact of juice turbidity on wine composition. This project aims to determine the influence of juice turbidity on fermentation rates, thiol production and wine composition in Sauvignon blanc wine.

Objectives:

- To investigate the effect of different levels of juice turbidity on fermentation rates and the impact on flavour and aroma profiles in the wine.
- Determine the extent to which juice turbidity influences wine composition during small scale winemaking using the Plant & Food Research (PFR) micro-vinification Sauvignon blanc winemaking protocols (700ml & 10L volumes).
- To identify the key juice turbidity measurement for optimum fermentation to achieve maximum flavour and aroma potential in the wine. Findings will verify winemaking protocols used within the Grape and Wine Research Programme.

Material and Methods

Sauvignon blanc grapes (three fruit lots of 60 kg) will be hand harvested from a commercial vineyard in Marlborough or alternatively commercial juice (90L) from the press will be sourced from a Marlborough winery.

- Harvested fruit will be stored overnight at 6^{o}C pre-processing or commercial juice will be collected from the winery and cold settled overnight.
- The 3 juice lots will be subsampled and 3 different juice turbidities (low, mid & high) applied by re-introducing a controlled amount of lees. 700mls of juice from each treatment will be fermented and replicated 3 times (27 wines).
- One larger ferment size (18L) will be subsampled from the 3 juice lots and 3 different juice turbidities (low, mid & high) applied as above (9 wines).

- Grapes are processed using the standard Plant and Food Research (PFR) winemaking protocol (see below).
- Fermentation rates will be monitored daily and samples taken for primary wine analysis and thiol and methoxypyrazine analysis at the end of fermentation. Dissolved oxygen concentrations will be monitored at key processing points (crush/destem, pressing and juice cold settling).

Marlborough Research Centre 8-10L

Sauvignon Blanc Winemaking Protocol 2015

1. Hand harvest 40kg fruit for each treatment from replicated vineyard plots. No field additions of sulphur.
2. Transfer to winery, crush and destem. Add PMS at 80g/T (40ppm SO_2).
3. Add enzyme (50ml/tonne Rapidase Clear (liquid)) to crushed/destemmed grapes.

   Enzyme addition: 0.5mls of 10% enzyme solution/kg.
4. Transfer to 25L containers under CO_2 cover. Give 1 hour skin contact time at 6˚C under CO_2 cover.
5. Transfer to hydro press. Collect free run under CO_2 cover. Press off under CO_2 cover. Pressing regime:
   i. Increase pressure slowly to 1.0 bar, hold for 2 min.
   ii. Increase pressure slowly to 3.5 bar, hold for 12 min.
6. Add 0.5g/L of bentinite slurry to pressed juice.
7. Cold settle juice for 24 hours at 6°C.
8. Rack off solids and transfer juice to 8-10L fermentation vessels.
9. Take 2 x microtubes and 1 x 50ml juice samples and freeze. 1 x 50ml falcon tube for primary juice analysis.
10. Warm juice to 15˚C and inoculate with X5 yeast (250mg/L), rehydrate yeast using standard procedure.

    Standard Yeast hydration procedure:

    - Heat water bath to 35-42˚C
    - Put required amount of distilled water in beaker and heat in water bath.
    - Check water temp with thermometer and when 32- 35˚C add yeast. Don't stir.
    - After 20 mins in water bath stir, remove and sit on bench for 15 mins. Put in bath of water in chiller to acclimatise to chiller temp.
    - Innoculate ferments (ensure ferments are inoculated within 45 mins from start of yeast rehydration)

11. Add DAP to a standard level (250ppm) if required, following a YAN analysis. Add DAP 1 day after inoculation.
12. Ferment at 15°C and aim for ferment temperatures of 14.5-15°C.
13. Ferment to dryness. Residual sugar ≤ 3g/L
14. Stop fermentation with 50ppm sulphur addition seal and settle.
15. Rack off gross lees 1 week after ferment has stopped.
16. Check SO_2 levels 1 week after stopping ferment (want molecular sulphur of 0.8ppm). Continue monitoring SO_2 levels monthly till bottling.
17. Filter (1.2 pre-filter and 0.45µm filter) and bottle. Take 4 x 50ml and microtube samples and freeze. Take 4 x 50ml falcon tubes for primary wine analysis

Base juice, Inoculation trial

Analysis	Inoculation rate Base juice
°Brix	18.8
Reducing sugars (g/l)	167,400
Glucose (g/l)	98,600
Fructose (g/l)	68,800
Potassium (mg/l)	1103
pH	3.07
TA (g/l)	8.6
Tartaric (g/l)	3.0
Malic (g/l)	4.0
YAN (mg/l)	207,71
Aas (mg/l)	149,31
NH_4 (mg/l)	58,40
Polyphenols (mg/l GAE) **	182,45
Folin Ciocalteu (mg/l GAE) **/***	3,7

Shows analysis of the base juice as used during the inoculation trial.

* ***A420/A320 is an oxidative index***

** ***GAE = Gallic acid equivalents***

*** ***Folin Ciocalteu = Winescan derived data***