



What is the Relationship Between the Use of Sulphur Dioxide and Biogenic Amine Levels in Wine?

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ABSTRACT

Vast research exists on biogenic amines (BAs) in food and beverages due to the toxicological impacts they have on health in addition to their negative quality implications. Symptoms of BA toxicity mirror those of wine intolerance, including headaches, nausea, rashes, and flushing. Yet most frequently, these symptoms are attributed to the additive sulphur dioxide (SO_2) even though clinical studies have established that reactions to SO_2 are almost exclusively respiratory, affecting 3–10% of the acute asthmatic population.

The effects of BAs in wine are heightened due to ethanol and acetaldehyde, which inhibit enzymatic detoxification. While decarboxylase-positive microbes, typically lactic acid bacteria (LAB), are fundamental to BA production, free amino acid precursors and conditions favourable to microbial growth must also be present. SO_2 has a well-known antimicrobial effect, yet its use in relation to BA accumulation has not been meaningfully studied. This study investigates the relationship between BAs and the use of SO_2 during vinification.

One hundred New Zealand Sauvignon Blanc wines were analysed for three BAs: histamine, tyramine and putrescine. A cross-section of wine styles and SO_2 regimes were represented: classic, alternative, low- and zero- SO_2 wines. There were highly statistically significant differences between SO_2 regimes and BA levels, with very high BA concentrations recorded in zero- and low- SO_2 wines while the lowest concentrations were recorded in the classic style.

Negative correlations between SO_2 and BAs demonstrate that both SO_2 amount and timing of addition (specifically pre alcoholic fermentation) are of critical importance to ensure BA levels remain below toxic thresholds in wines, exposing the irony of the

widely held view on SO₂ and wine intolerance. The argument for establishing a low-BA category of wine is compelling.

1. INTRODUCTION

Biogenic amines (BAs) are a group of low-molecular-weight compounds chiefly derived from the decarboxylation¹ of the corresponding amino acids (AA) by specific microbes² (ten Brink et al. 1990). BAs are formed and degraded through normal metabolic activity. Their biological genesis means they can accumulate in many foods and beverages (F&B) due to the presence of decarboxylase-positive microorganisms, primarily bacteria, which are especially common in fermented products. In foods such as meat and fish, BA presence is used as a quality index as at high levels they suggest spoilage has occurred (Silla-Santos 1996).

BAs have manifold roles in normal human physiological function, including neurotransmission, immune modulation, circadian rhythm, respiratory function and cardiovascular activity. However, excessive oral intake of BAs can cause adverse reactions such as nausea, headache, rhinoconjunctival symptoms, hyper- or hypotension, flushing, rashes and heart palpitations due to the complex pharmacological effects associated with the histamine and neurotransmitter systems (Maintz and Novak 2007). Sensitivity among individuals varies because of genetic or gastrointestinal conditions that impair enzymatic function (Silla-Santos 1996). Moreover, the body's ability to enzymatically metabolise BAs can be prevented by amine oxidase inhibitors of which ethanol is one of the most potent (Zimatkina and Anichtchik 1999). Consequently, BA levels in wine are even more significant.

¹ Decarboxylation is a chemical reaction that removes a carboxyl group (a carbon atom double-bonded to an oxygen atom) from a molecule, thereby releasing carbon dioxide (Biology Online, 2020 available online: <https://www.biologyonline.com/dictionary/decarboxylation>)

² To a lesser extent BAs can also be derived from animation and transamination of aldehydes and ketones (Smit et al. 2008).

The presence of BAs in wine has been abundantly researched yet on a practical level neither the industry nor consumers at large are aware of their relevance to health or to product quality. Quality is affected by microbial spoilage and potential sensory effects (Smit et al. 2008), while the most prolific BA-producing bacteria are also implicated in organoleptic faults such as mousiness and ropiness (Costello 1998, Lonvaud-Funel 2010). BAs in wine are not presently subject to legal regulation.

Numerous factors influence BA levels in wine, but it has been established that the most quantitatively and qualitatively important are winemaking practices (Herbert et al. 2005). At a macro-level these are practices that i) increase AA precursors and ii) promote microbial growth in general. However, decarboxylase-positive microbes must be present.

BA levels in wines have been analysed with many variables examined, including geographical origin, variety, vintage and some winemaking practices, yet no studies have focused primarily on the influence of sulphur dioxide (SO_2). Free SO_2 (FSO_2) is a potent microbial inhibitor but bound SO_2 is anti-bacterial, with even small amounts shown to be effective against wine bacteria, especially those that are the most prolific BA-producing microbes (Wells and Osborne 2012). The effective use of SO_2 and specifically the timing of addition(s) could therefore be a major tool in reducing BA levels by inhibiting the growth of decarboxylase-positive microbes.

This is cast against a widely held, but misinformed, belief among consumers and media alike that adverse reactions to wine consumption are caused by SO_2 , especially when alcohol as a causative agent can be eliminated. However, sulphite

reactions are almost exclusively limited to respiratory response, rather than the full suite of symptoms that BAs induce (Vally and Misso 2012).

The apparent contradiction is underscored by the current movement in the global industry towards low-/zero-SO₂-addition wines. Meanwhile, the risks that BAs pose to public health remain unknown and unseen. Ethanol's inhibitory effect on BA detoxification compounds the hazard.

It is beyond coincidence that symptoms of BA toxicity mirror the anecdotal evidence for adverse reactions from wine consumption. This paper will therefore review existing research on wine intolerance before outlining BA functions and impacts on health. The study will explore BAs in wine and examine how BA accumulation is influenced by winemaking practices focusing on SO₂ regime, including amount and timing of addition(s).

2. LITERATURE REVIEW

While BAs are the focus of this review, it is necessary to briefly address the subject of wine intolerance to establish the context for the consideration of BAs in wine.

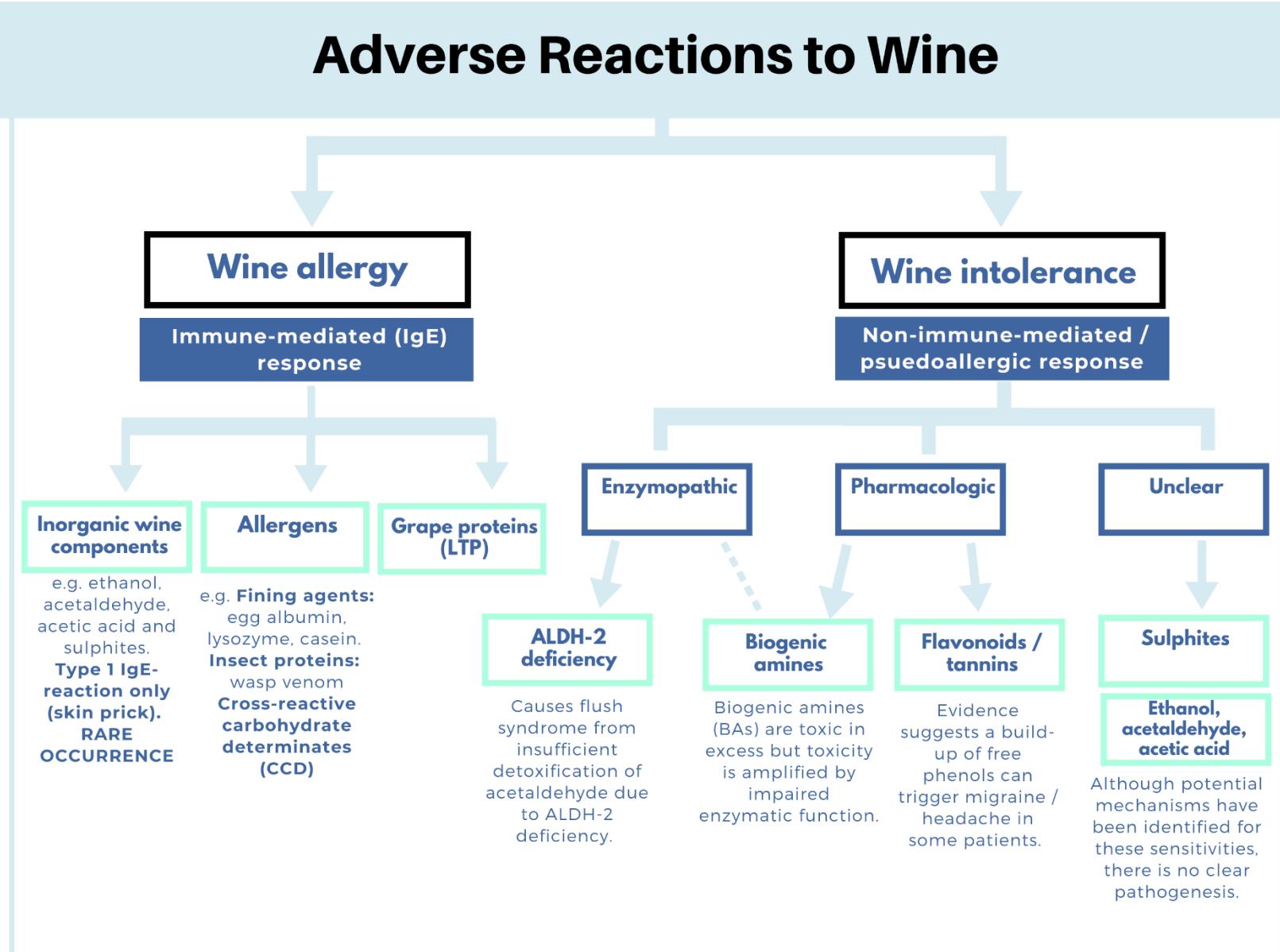
2.1. The mysteries of wine intolerance

Adverse reactions to wine or ‘wine intolerance’ have been both clinically studied and anecdotally reported with myriad potential causes proffered. The complexity of wine, with 600-odd identified components, seemingly justifies this. Recently, greater public interest in wine and its constituents has resulted from the emergence of movements such as Clean Wine and Natural Wine. Compounding this is the attention of mainstream media and stakeholders seeking to fulfil their customers’ desires for healthy, natural products.

Just two epidemiological studies on wine intolerance have been undertaken on a general population (Wigand et al. 2012, Linneberg et al. 2008). Wine intolerance was more common than expected, with 7–8% experiencing it frequently. Reinforcing this, an allergy clinician estimates 1/10 patients referred in general either have a history of reactions to alcohol or report alcohol as an aggravator of a pre-existing allergy, with wine the most common trigger (McGettigan 2020 pers. comm.).

Wine intolerance is distinct from wine allergy, which is an immunoglobulin E (IgE)-mediated response and is relatively uncommon (Wigand et al. 2012). Intolerance reactions are a non-immune-mediated sensitivity and potentially include reactions to ethanol, acetaldehyde, flavonoids, sulphites and BAs (Wüthrich 2018). Figure 1 demonstrates this distinction.

Adverse Reactions to Wine



Adapted from Wüthrich 2018.

Figure 1 - Categorisation of adverse reactions to wine

Although wine intolerance is not an allergy, individuals anecdotally claim they are ‘allergic to wine’ because of their allergic-type response, hence the classification pseudoallergic (Wüthrich 2018). Symptoms are diverse, with the general consensus that if two or more of the following are present, it is an instance of wine intolerance: circulatory collapse; shortness of breath/asthma; tachycardia; itching; flushed skin; swelling of the lips, mouth, throat; low blood pressure; rhinorrhoea; burning sensation in the lips, palate, neck; stomach or intestinal cramps; diarrhoea; vomiting; headache (Wigand et al. 2012).

Anecdotally, wine intolerance is most frequently attributed to the additive SO₂. The Clean Wine and Natural Wine movements promote the avoidance of SO₂ or its limited use as an additive as a key principle in their philosophies, implying SO₂’s negative health impacts (Good Clean Wine 2020, Legeron 2014). This is reflected in consumer attitudes toward SO₂; a study by Costanigro et al. (2014) found 34% of their 223-sample size experienced headaches after moderate wine consumption, and SO₂ was the most frequently attributed cause. McGettigan states there is more anxiety around sulphites than other factors such as BAs. This appears driven by mainstream media. Patients were more likely to misassign symptoms to SO₂ when there were likely other factors at play (McGettigan, 2020 pers. comm.).

Studies have shown that sulphite sensitivity is confined to a small percentage of the acute asthmatic population. A range of 3–10% of globally diagnosed asthmatics is generally accepted (Vally and Misso 2012), while in non-asthmatics sensitivity is rare (Bush 1986, Lester 1995). The severity of reactions varies (from mild to, rarely, life-threatening), with a high correlation in sensitivity seen in those who require daily oral corticosteroids and who have a known sensitivity to other sulphite-containing foods/drugs (Vally and Misso 2012). Furthermore, Vally and Thompson (2001)

demonstrated that sensitivity to SO₂ was potentially overestimated after single- and cumulative-dose challenges failed to show a significant correlation between SO₂ and asthmatic response.

Most wine intolerance complaints, including from asthmatics, arise after the consumption of red wine, which is contrary to the reality of relative SO₂ levels: white wines and sparkling wines typically contain more than red. Revealingly, asthmatoïd wheezing/bronchoconstriction is a symptom of BA toxicity (Wantke et al. 1996, Wöhrl et al. 2004), therefore it is possible that the wine intolerance some asthmatics experience is in fact from BA-sensitivity. This could potentially explain the phenomenon of red-wine provoked asthma (Wantke et al. 1996, Wantke et al. 1994) as BA levels are typically higher in red wines, but SO₂ levels relatively low when compared with those of white wines. Maintz and Novak (2007) drew attention to a potential genetic link between white asthmatic patients and reduced histamine metabolism.

Sulphite-sensitive subjects have reported other symptoms (dermatitis, urticaria, flushing, abdominal pain and diarrhoea) but these are rare, with symptoms almost exclusively respiratory (Vally and Misso 2012). No link has been conclusively established between SO₂ and headache (Panconesi 2008).

Other components of wine including alcohol itself have been suggested as causes of wine intolerance, but detailed consideration is beyond this review's scope. Briefly, Panconesi (2008) found no significant association between alcohol and migraine/tension headache (distinct from hangover headache), and that excessive quantities did not need to be ingested to produce headache. Breslin et al. (1973) found no conclusive link between alcohol and bronchial/asthmatic reactions.

Both flavonoid phenols in wine and the release of 5-hydroxytryptamine³ have been proposed as potential migraine-inducing mechanisms, and although plausible for red-wine-induced headache, few conclusive studies exist (Panconesi 2008).

2.2. Biogenic amines

It is well established in the literature that BAs can compromise F&B safety (Ruiz-Capillas and Herrero 2019). This section will discuss BA production, function, and toxicity in general before reviewing the factors influencing BA formation and accumulation during vinification (section 2.3).

2.2.1. BA production

BAs are organic bases of low molecular weight, formed and degraded by the normal metabolic function of living organisms (ten Brink et al. 1990). They are both fundamental and potentially detrimental to health. While BAs are involved in essential biological processes, they become toxic to humans if they exceed basal levels (ten Brink et al. 1990). The most common sources of exogenous BAs for humans are F&B that contain high concentrations (Silla-Santos 1996).

BAs are most frequently formed from their equivalent AA precursors and the BA name is often derived from this. Numerous BAs have been identified and several classifications exist depending on the BA's functions and properties. Table 1 lists common BAs alongside their structures, types and AA precursors.

³ 5-hydroxytryptamine, or serotonin, is a biogenic amine, specifically a monoamine neurotransmitter (Medical Dictionary, The Free Dictionary, 2020 available online: <https://medical-dictionary.thefreedictionary.com/5-hydroxytryptamine>)

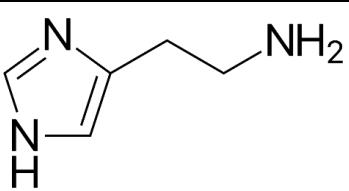
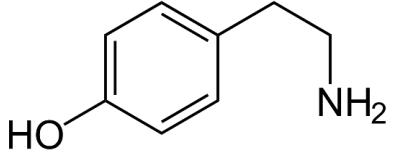
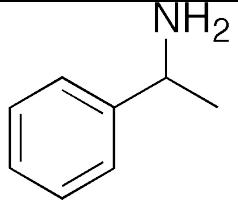
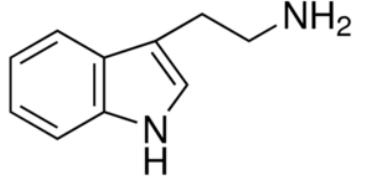
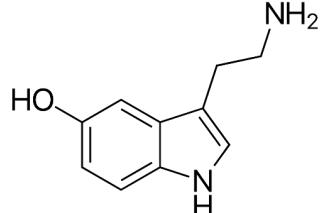
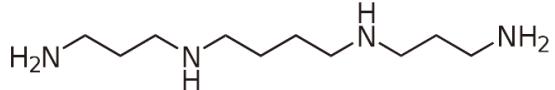
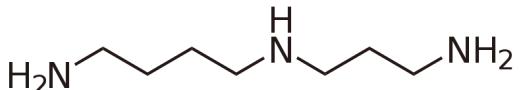
Biogenic amine	Structure	Type	Amino acid precursor
Histamine		Heterocyclic Non-Volatile Monoamine	Histidine
Tyramine		Aromatic Non-Volatile Monoamine	Tyrosine
Putrescine		Aliphatic Volatile Polyamine	Ornithine Arginine (alternative decarboxylation pathway (Smit et al. 2008))
Cadaverine		Aliphatic Volatile Polyamine	Lysine
Phenylethylamine		Aromatic Volatile Monoamine	Phenylalanine
Tryptamine		Heterocyclic Non-volatile Monoamine	Tryptophane
Serotonin / 5-Hydroxytryptamine (5-HT)		Heterocyclic Non-volatile Monoamine	Tryptophane
Spermine		Aliphatic Volatile Polyamine	Ornithine (synthesised from Spermidine)
Spermidine		Aliphatic Volatile Polyamine	Ornithine (synthesised from Putrescine)

Table 1 - Common BAs, their structure, type, and amino acid precursor

Over 20 BAs have been identified in wine (Lehtonen et al. 1992) with histamine, tyramine and putrescine the most common (Soufleros et al. 1998). Cadaverine, phenylethylamine, spermidine and tryptamine are less common and found in lower concentrations (Russo et al. 2010, Silla-Santos 1996). Histamine and tyramine are widely recognised as the most toxic BAs to humans when ingested above the levels that can be metabolised (EFSA 2011).

There are three overarching prerequisites for appreciable production of BAs (ten Brink et al. 1990), which are explored in the context of vinification in 3.3. These are:

- 1) Availability of free AAs or proteins⁴
- 2) The presence of microorganisms with decarboxylase genes
- 3) Conditions that favour microbial growth and decarboxylase synthesis/activity.

BAs have been found in a wide range of products, with high concentrations found in fish, cheese, soybean products, chocolate, sausages, processed meat, wine and beer (Costantini et al. 2019). All products that possess proteins and/or free AAs and have microbial/biochemical activity can theoretically accumulate BAs. This is especially true of fermented products. The total BA content and the type of BAs present depends upon the product's nature and the attendant microorganisms (ten Brink et al. 1990), as well as the microbial activity occurring throughout the production, storage and distribution chain. Presence of BAs in F&B is considered undesirable as it is a strong indicator that microbial spoilage has occurred (Silla-Santos 1996).

⁴ Proteolytic activity liberates AAs from proteins (Leitao et al. 2000).

2.2.2. Function, toxicity and detoxification

Ingestion of BAs above basal levels⁵ can trigger a range of symptoms (Maintz and Novak 2007), collated in Table 2. Histamine and its effects are perhaps best known because of its role as a mediator in allergic disorders and because in most cases of toxicity it is histamine that is implicated (Taylor et al. 1989). The release of histamine in response to an allergic reaction and the consumption of histamine-containing food exerts the same physiological effects (ten Brink et al. 1990).

Because of the pseudoallergic nature of BA toxicity, symptoms can often be misdiagnosed as an allergy but it is the cumulative ingestion of BAs that lead to toxicity, rather than the symptoms being elicited by a very small amount of the allergen (Maintz and Novak 2007). As can be seen, there is a strong correlation between symptoms of BA toxicity and commonly reported wine intolerance reactions.

When low concentrations of BAs are ingested, the body has a natural defence system to eliminate them. The intestines limit the absorption of BAs through the intestinal mucin layer and the closely linked epithelial cells, but BAs are also detoxified in the body by the action of enzymes HNMT⁶ and di-amine oxidase (DAO) (Maintz and Novak 2007). DAO is the main enzyme responsible for metabolism of ingested histamine but is not located in the brain, having possible relevance to BA-induced headaches (Jarisch et al. 1996). Women have lower levels of DAO than men, consistent with anecdotal observations of gender prevalence in wine intolerance (Jarisch et al. 1996).

⁵ The basal level of histamine in a normal, healthy individual ranges from 0.3 to 1 µg/mL (Maintz and Novak 2007)

⁶ HNMT also involves the sub-enzyme of mono-amine oxidase (MAO), which assists HNMT in breaking histamine down further into other metabolites (Skilton 2019).

Biogenic amine	Function	Toxicity symptoms
Histamine	<ul style="list-style-type: none"> Neurotransmitter and vasodilator on the central nervous and cardiovascular systems. Dilates peripheral blood vessels, capillaries and arteries. Excites smooth muscles of the uterus and gastrointestinal tract, enhancing acid secretion from the gastric mucosa. Stimulates mucus secretions and causes endothelial permeability of the airway. 	<ul style="list-style-type: none"> Migraine/headache, vertigo, hypotension, flushing, pruritus (itching), urticaria (hives), arrhythmia, tachycardia. Nausea, vomiting, dysmenorrhea, cramps, stomach ache, diarrhoea. Nasal congestion, rhinorrhoea sneezing, wheezing, bronchoconstriction, anaphylaxis.
Tyramine	<ul style="list-style-type: none"> Promotes catecholamine efflux from the sympathetic nervous system and the adrenal medulla. Dilates pupils and palpebral tissue, causes lacrimation and salivation, accelerates respiration and increases blood sugar content. 	<ul style="list-style-type: none"> Increase in mean arterial blood pressure and heart rate by peripheral vasoconstriction – migraine/headache, hypertension. Has been linked to development of Parkinson's disease, schizophrenia and mood disorders.
Phenylethylamine	<ul style="list-style-type: none"> Regulates monoamine neurotransmission. Neurotransmitter in the human central nervous system. 	<ul style="list-style-type: none"> Hypertension, headache/migraine.
Polyamines – putrescine, cadaverine	<ul style="list-style-type: none"> Involved in cell proliferation. Formation of carcinogenic nitrosamines by reaction between nitrite and secondary amines (putrescine, cadaverine, agmatine). 	<ul style="list-style-type: none"> Carcinogenesis, tumour invasion.

Adapted from Maintz and Novak (2007), Smit et al. (2008).

Table 2 - Biogenic amine function and symptoms of toxicity

The dose-response relationship is not straightforward, and the literature reflects this.

Complicating factors include:

- i) **Individual sensitivity:** the ability to detoxify BAs varies among individuals because certain genetic predispositions or gastrointestinal conditions/disease impair enzymatic function and/or affect absorption (Maintz and Novak 2007).
- ii) **Inhibition of DAO/HNMT:** some substances can inhibit DAO/HNMT. One of the most competitively active inhibitors of DAO is ethanol (Zimatkina and Anichtchik 1999). Acetaldehyde and certain DAO/HNMT-inhibiting drugs (DAOIs/MAOIs) also decrease function (Maintz and Novak 2007). The largest subgroup of DAOIs/MAOIs are antidepressants (see Appendix 2 for an extensive list). 20% of Europe's population regularly takes DAOI/MAOI antidepressants (Ruiz-Capillas and Herrero 2019).
- iii) **Co-potentiating effect of BAs:** certain BAs, particularly polyamines, have been shown to have a co-potentiating effect on histamine and tyramine. Bulush et al. (2009) confirmed that in the presence of cadaverine and putrescine, histamine-metabolising enzymes were inhibited. Histamine toxicity increased with BA co-potentiators by depressing histamine oxidation (Ibe et al. 1991).

Furthermore, approximately 1% of the population has clinically recognised histamine intolerance (enteral histaminosis), which is a disequilibrium of accumulated histamine and an inability to degrade it effectively (Maintz and Novak 2007). These are considered histamine-sensitive individuals and the condition is distinct from histamine toxicity resulting from ingestion of toxic amounts.

2.2.3. Toxicity thresholds

Although the literature has attempted to establish general toxicity thresholds for BAs, there has been little agreement. Much of the focus is on histamine in fish products⁷ as this is the leading identified cause of histamine toxicity (Taylor et al. 1989, EFSA 2011).

The clinical studies on BA toxicity in the context of wine consumption are problematic for establishing a toxicity threshold as they:

- i) All fail to consider the complicating factors, such as:
 - a. ethanol's role as a contributor (most studies have a range of 125–200 mL total wine volume ingested),
 - b. the co-potentiating effects of BAs (only histamine is studied, with tyramine a variable in one study).
- ii) Do not have robust scientific design, primarily:
 - a. are not double-blind placebo-controlled (DBPC) (Wantke et al. 1994, Lüthy and Schlatter 1983, Dahl et al. 1986), and/or
 - b. have a high response to the placebo, invalidating results (Kanny et al. 2001, Lüthy and Schlatter 1983), and/or
- iii) focus exclusively on histamine-sensitive subjects (Menne et al. 2001, Wantke et al. 1994) or asthmatic patients (Dahl et al. 1986), failing to consider the impact of ordinary wine-consuming occasions on healthy, moderate wine drinkers.

⁷ Most at risk fish products are those from the Scombridae/Scomberesocidae family which include mackerel, tuna and bonito. However, certain non-scombroid fish, most notably mahi-mahi, bluefish and sardines are, when spoiled, also commonly implicated in histamine toxicity (Taylor et al. 1989).

There is just one clinical study that is acceptable for its scientific design (Menne et al. 2001). It was DBPC but did not consider co-potentiating factors and included histamine-sensitive subjects only. It found 20 mg/L histamine in 200 mL of sparkling wine consumed (4 mg total dose) triggered symptoms in 12/40 patients. Appendix 3 overviews and critiques studies on BA toxicity in wine.

Esposito et al. (2019) is a case study of suspected BA toxicity from BA-rich wine. It is the first study of its kind to focus on dietary exposure to BAs in wine in a non-clinical setting. Six young healthy individuals experienced BA toxicity symptoms after consuming a wine on tap in Italy. When BA toxicity was suspected, the same wine was analysed, with high levels of BAs recorded: histamine, tyramine and putrescine totalled 9.97, 8.23 and 13.01 mg/L respectively. The wine volume consumed was approximately three glasses (~400 mL), which equated to total dose amounts of 4 mg histamine, 3.3 mg tyramine and 5.2 mg putrescine. This is the only source of BA toxicity thresholds in wine in a non-clinical context.

These two studies agree on a toxicity threshold for histamine (4 mg) but evidently more robustly designed clinical studies with conditions simulating normal wine-consuming occasions are required.

Despite acknowledging the complexity of BA accumulation in fermented F&B and the product-specific nature of BA interactions including co-potentiating factors, the European Food Safety Authority (EFSA 2011) indicated a blanket limit of 25–50 mg histamine per person per meal as a no-observed-adverse-effect-level (NOAEL). This limit has since been applied by numerous authors to determine that wines studied with relatively high BA levels would in fact be safe for consumption. Yet the EFSA considered both a limited number of studies ($n=2$) and individuals (healthy $n=20$;

sensitive $n=40$) to evidence dose–response relationships in wine (Menne et al. 2001, Lüthy and Schlatter 1983). The latter’s high response to the placebo makes these results inconclusive.

Histamine toxicity has been established intravenously at 0.2–0.5 mg, which caused healthy subjects ‘violent and throbbing headaches’, along with increased pulse rate, flushing, dizziness, vomiting and diarrhoea, while 3–8 mg caused toxicity when administered subcutaneously (Schneyder 1973).

Limited literature exists on toxicity thresholds in wine of other BAs, such as tyramine and polyamines. The EFSA (2011) cited insufficient information to establish a NOAEL for tyramine. The only study including tyramine is inconclusive because of inadequate blinding (Littlewood et al. 1988).

Nonetheless, occurrence of non-IgE-mediated reactions to F&B products is common. Ideally DBPC provocations would be performed on all patients to determine individual thresholds, but time and cost prevent their adoption in clinical practice (Maintz and Novak 2007, McGettigan 2020 pers. comm.)

Consequently, the prevailing advice in the literature and echoed by food safety authorities is for F&B manufacturers to take all steps possible to minimise BA accumulation as a matter of public health and safety. This reinforces Zee et al. (1983), who surmised that because of the variation in individual susceptibility to BAs, there is no minimum amount of BAs that must be ingested before toxic effects are observed.

Presently there are no legally regulated BA limits for wine in any country.⁸ Some countries have recommended upper limits but for histamine only (Table 3).

Country	Recommended upper histamine limit
Germany	2 mg/L
Netherlands	3.5 mg/L
Belgium	6 mg/L
Finland	5–9 mg/L
France	8 mg/L
Switzerland	10 mg/L
Australia	10 mg/L

Adapted from Restuccia et al. (2018).

Table 3 – Recommended upper histamine limits in countries worldwide

Therefore, this recommendation has not *prima facie* been embraced by the wine industry and its relative inaction on addressing BAs in its products, unlike industries such as dairy and seafood, is apparent. While the high ethanol/acid environment of wine makes it inhospitable to the most pernicious microorganisms, it is evident that BA presence in wine is indeed a demonstrable threat to human health and safety that needs consideration and management.

⁸ Switzerland had established an official maximum limit of 10 mg/L for the presence of histamine in wines but in 2011 ceased enforcing this restriction for imported wines (Restuccia et al. 2018). No reason was given for eliminating the limit.

2.3. Biogenic amines and vinification

Many studies exist on the influence of winemaking practices on BA formation. Most focus on microbial activity as the key driver of BA accumulation. Two primary origins of BAs have been identified: raw materials and winemaking processes. There is consensus that the latter are qualitatively and quantitatively more important for BA accumulation (Herbert et al. 2005).

The prerequisites for BA accumulation are relevant to the winemaking chain, just as they are to other industries (Figure 2 and outlined in section 2.2.1).

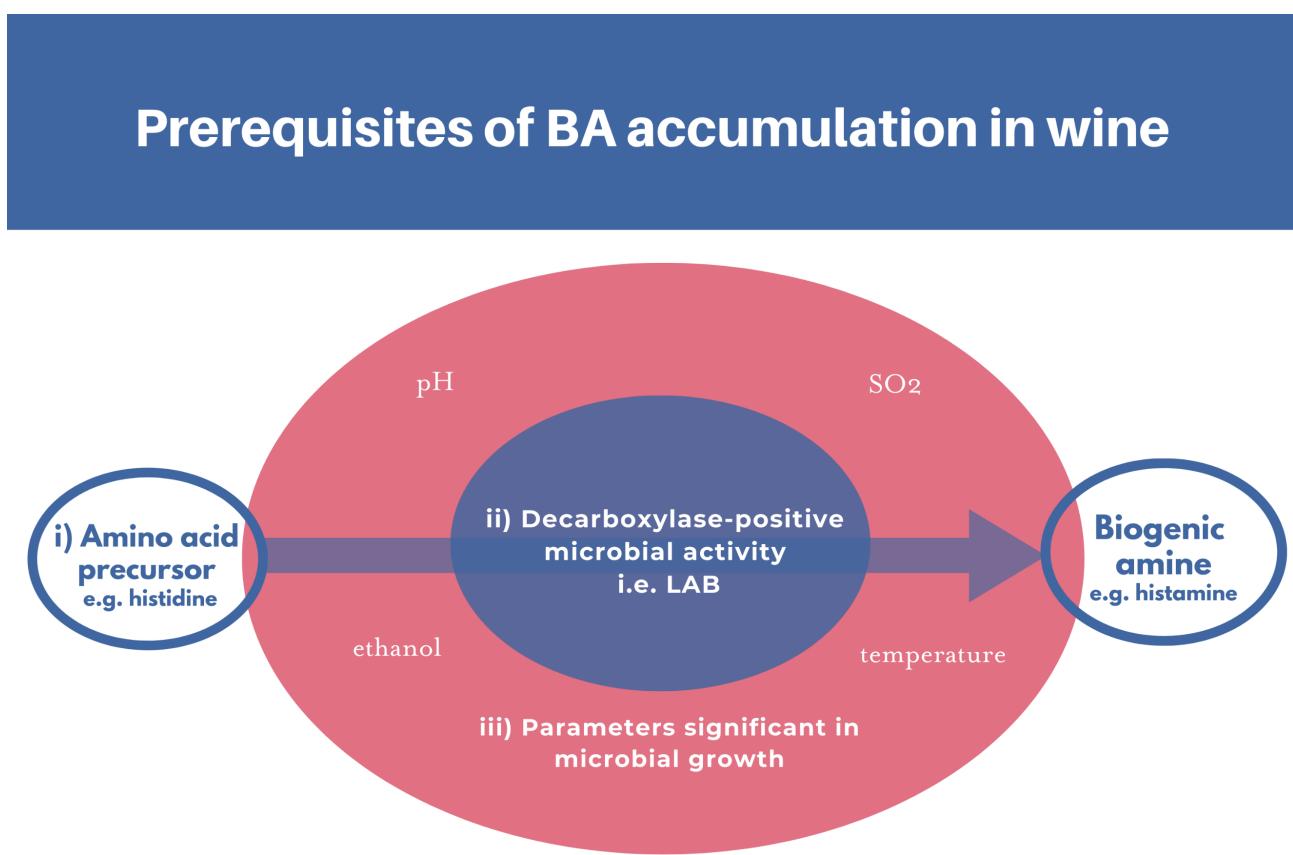


Figure 2 - Prerequisites of BA accumulation in wine

Decarboxylase-positive microbes must be present in the medium to convert the AA precursor into the respective BA. In the case of wine, it is essentially lactic acid bacteria (LAB) that carry out this activity. While some authors have found certain

yeasts to be capable of synthesising BAs, their net contribution to BA production is relatively minor, producing negligible amounts of the most toxic BAs (Herbert et al. 2005, Marcabal et al. 2006). One exception is the spoilage yeast *Brettanomyces bruxellensis*, which, among yeasts, produces the highest amounts of BAs (Caruso et al. 2002, Granchi et al. 2005). Acetic acid bacteria studied have not produced BAs (Landete et al. 2007).

Bacteria are always present in wine whether naturally occurring or added. However, it is wine parameters such as pH, ethanol content, temperature and SO₂ concentration that will determine their population and BA synthesis. This key notion is often neglected in the literature, which instead prioritises winemaking practices over these parameters. While it is true that BA levels tend to increase as AA levels increase and that decarboxylase-positive microbes are essential for BA formation, if conditions are not hospitable for LAB growth and activity, BA accumulation will be restricted.

Only the most significant factors influencing BA accumulation in winemaking will be discussed here, beginning with microbial activity, followed by AA precursors and, finally, wine parameters.

2.3.1. Microbial activity

Yeasts

There is general agreement that yeasts' contribution to BA content in wine is less than that of LAB. Some authors have found no significant increase or even a decrease in BA levels during alcoholic fermentation (AF) using either ambient or commercial yeast strains (Herbert et al. 2005, Marcabal et al. 2006, Landete et al. 2007). Several studies have found certain yeasts can produce BAs (Buteau et al.

1984, Goñi and Ancín-Azpilicueta 2001, Torrea and Ancín 2002, Caruso et al. 2002, Granchi et al. 2005, Tristezza et al. 2013), but in all instances histamine and tyramine were not detected (ND) or at a low level (<4 mg/L).

S. cerevisiae's ability to produce BAs is significantly strain dependent, with these strains producing ethanolamine and agmatine in quantities up to 10 mg/L (Caruso et al. 2002). *Brettanomyces bruxellensis* produced the highest total BA concentration among yeasts in the two studies that considered it. 2-phenylethylamine was the predominant BA in both cases (Granchi et al. 2005, Caruso et al. 2002). Landete et al. (2007) found LAB to be exclusively responsible for BA accumulation in wine, demonstrating that of 231 microorganisms studied, neither yeasts ($n=36$) nor acetic acid bacteria ($n=40$) could produce BAs. However, this paper did not include *Brettanomyces* spp. Yeast is therefore a minor factor in BA accumulation.

LAB

Not all LAB are equal in their ability to produce BAs as they must possess BA-specific decarboxylase genes, which are species and strain dependent (Smit et al. 2008). The enzymes histidine decarboxylase (HDC), tyrosine decarboxylase (TDC) and, for putrescine, ornithine decarboxylase (ODC) or the arginine/agmatine pathway are the principal mechanisms for AA to BA conversion (Ancín-Azpilicueta et al. 2008).

Oenococcus, *Leuconostoc*, *Lactobacillus* and *Pediococcus* spp. have all been found to produce a variety of BAs in wine (Smith et al. 2008). Table 4 details the species/strains with identified BA-decarboxylase activity to date. *Lactobacillus hilgardii* and *Pediococcus parvulus* spp. have been identified as the most prolific

histamine producers (up to 200 mg/L concentrations) and the former is also a strong tyramine producer (Landete et al. 2005b).

Many authors assume that LAB's role in malolactic fermentation (MLF) means MLF contributes the greatest BA concentrations. Most studies therefore examine the impact of MLF on wine BA levels, yet the research establishes that LAB's ability to produce BAs is mostly distributed among non-*O. oeni* LAB species that do not habitually perform MLF. Therefore, the focus on MLF seems theoretically misguided; MLF is a factor, but not essential, for significant BA accumulation in wine. Lonvaud-Funel and Joyeux (1994) reinforced this by showing that histidine decarboxylation was enhanced when malic acid was excluded from the medium, meaning MLF did not occur. So, while it is not the metabolic process of MLF itself that produces BAs, the conditions necessary for MLF augment the growth of all LAB that can produce BAs.

Studies typically show that spontaneous MLF produces statistically higher BA levels than inoculated MLF (Martin-Alvarez et al. 2006). This can be attributed to non-*O. oeni* LAB being involved and the fact that commercial MLF starter cultures are selected to avoid decarboxylase-positive LAB. Accordingly the universal recommendation for minimizing BA accumulation is to inoculate with LAB starters immediately after AF or to co-inoculate rather than allow spontaneous MLF (International Organisation of Vine and Wine (OIV) 2011). By contrast, spontaneous MLF is typically the preferred technique in most premium wines that undergo MLF. Moreover, while MLF is the principal indication that LAB are abundant, the strain-specific nature of LAB's BA-producing ability may go some way to explaining the few papers that found a negative correlation between MLF/LAB and BA accumulation (Buteau et al. 1984, Ough et al. 2007).

Lactic acid bacteria (LAB)		Decarboxylase enzymes		
Genera	Species	Histidine decarboxylase (HDC)	Tyrosine decarboxylase (TDC)	Ornithine decarboxylase (ODC) or arginine pathway for putrescine
<i>Oenococcus</i>	<i>O. oeni</i>	Highest frequency of histamine production but produced the lowest concentrations (Landete et al. 2005b)	Can produce tyramine but has a low activity to do so (Moreno-Arribas et al. 2000, Guerrini et al. 2002, Choudhury et al. 1990)	Although putrescine most prolific BA in wine, very few identified strains possess ODC. BIFI-83 strain isolated from lees of Spanish wine (Marcobal et al. 2004). 7/44 strains studied able to produce PUT in a culture media. IOEC 8419 strain isolated from wine, capable of producing putrescine (Coton et al. 1999)
<i>Lactobacillus</i>	<i>L. hilgardii</i>	Produced histamine at the highest concentrations – up to 200mg/L (Landete et al. 2005b)	Strong tyramine producer. IOEB 9649 strain can simultaneously produce tyramine and phenylethylamine (Moreno-Arribas et al. 2000)	
	<i>L. malii</i>	Histamine producer (Landete et al. 2005b)		
	<i>L. buchneri</i>			Two strains could produce putrescine via ODC pathway. (Moreno-Arribas et al. 2003)
	<i>L. brevis</i>		Strong tyramine producer (Moreno-Arribas et al. 2000) IOEB 9809 can simultaneously produce tyramine and phenylethylamine (Moreno-Arribas et al. 2000)	
	<i>L. plantarum</i>		TYR producer (Bonnin-Jusserand et al. 2012)	Capable of producing putrescine from both arginine and ornithine (N4 strain) (Arena and Manca de Nadra 2001)
	<i>L. saerimneri</i>			L 30a strain possessed ODC gene (Gale 1946, Tabor and Tabor 1985)
<i>Pediococcus</i>	<i>P. parvulus</i>	Produced histamine at the highest concentrations – up to 200mg/L (Landete et al. 2005b)		
<i>Leuconostoc</i>	<i>Leuc. mesenteroides</i>	Can produce high levels of histamine. (Landete et al. 2007b)	Has a high potential to produce tyramine (Moreno-Arribas et al. 2003)	

Table 4 - Lactic acid bacteria studied in literature showing decarboxylase activity and BA production

Post-MLF ageing

Several studies have found BAs increase in the maturation period after MLF and in bottle (Herbert et al. 2005, Gerbaux and Monamy 2000, Jiménez-Moreno et al. 2003, Henríquez-Aedo et al. 2018, Marques et al. 2008). Coton et al. (1998) demonstrated that enzyme activity can outlast the bacteria population during lees ageing by several months. This suggests it is possible for BAs to accumulate even in the absence of viable LAB in wine (Ancín-Azpilicueta et al. 2008). Polo et al. (2011) found that the longer the LAB population remained during the ageing process, the higher the total BA levels.

2.3.2. Factors influencing free AAs

It has been found that AA content can be influenced by vinification practices, grape variety, geographical region and vintage (Lonvaud-Funel and Joyeux 1994, Soufleros et al. 1998, Moreno-Arribas et al. 2000). It is important to remember that free AAs do not automatically equate to higher BA levels.

The limited scope of this paper necessitates focusing on vinification practices only. The most consequential are reviewed below.

Skin contact

As grape skins contain AAs, it follows that extended contact of must/wine with skins can lead to BA formation if decarboxylase-positive microbes are present. It is generally agreed that skin contact, unavoidable for red-wine vinification, contributes to red wines' typically higher BA levels (Zee et al. 1983).

Most papers examining maceration time found a positive correlation with BA accumulation (Bauzá et al. 1995, Martin-Alvarez et al. 2006, Kovačević Ganić et al.

2009). Kovačević Ganić et al. (2009) studied skin contact in white wines as a variable. Of three different vinifications (free run, press wine and macerated wine), macerated wine had the highest resulting BA concentrations.

Lees contact and ageing

Time on lees enables microbial conversion of proteins to AAs (Lonvaud-Funel and Joyeux 1994). Several authors have found >3 months lees ageing increases BA levels (Martin-Alvarez et al. 2006, Gonzalez-Marco and Ancín-Azpilicueta 2006b, Marques et al. 2008).

Lees stirring

Lees stirring increased the BA content of wines, especially histamine in red wines, and tyramine in Chardonnay. Highest concentrations resulted from weekly stirring (Alcaide-Hidalgo et al. 2007, Gonzalez-Marco and Ancín-Azpilicueta 2006b).

Nitrogen-containing supplements

In Garcia-Marino et al. (2010), addition of yeast mannoproteins resulted in an increase in BAs. This was attributed to a rise in nitrogen compounds that could be used as AA precursors. Marques et al. (2008) found that nitrogen-containing supplements to aid fermentation did not appear to influence BA formation.

2.3.3. Wine parameters

Several factors profoundly influence the concentration and diversity of microorganisms in the wine as well as affecting decarboxylase activity. These are interrelated.

pH

There is a strong correlation between pH and BA levels. High pH typically equates to higher BA levels while lower pH reduces BA formation (Lonvaud-Funel and Joyeux 1994, Gardini et al. 2005, Martín-Álvarez et al. 2006). Landete et al. (2005a) found histamine increased significantly when pH was above 3.6.

The higher the pH, the greater the diversity of bacterial microflora (including spoilage bacteria) and the more likely the growth and survival of decarboxylase-positive bacteria (Lonvaud-Funel 2001). Wines below pH 3.3 become inhospitable to LAB, and MLF becomes more difficult, thus lower BA levels are likely but not guaranteed.

There is an intrinsic link between pH and SO₂, as the latter becomes less effective at higher pH (see section 5.2).

Ethanol

While ethanol has not been studied as a parameter in itself, it has been found that high ethanol concentrations (>12% abv), combined with either low pH or low pyridoxal 5-phosphate⁹ concentrations produced lower BA levels (Gardini et al. 2005). Lonvaud-Funel and Joyeux (1994) showed that BA concentrations were the highest in wine with high pH and low ethanol, but that pH was the most decisive factor in all cases.

SO₂

One of the most overlooked factors in BA accumulation is SO₂ use during vinification.

⁹ Pyridoxal 5-phosphate is the active form of vitamin B₆ and is a coenzyme in a variety of enzymatic reactions, including all transamination reactions, and certain decarboxylation and deamination reactions of amino acids (Dolphin et al. 1986).

The toxicity of FSO₂ and bound SO₂ to LAB is well-established, yet research on the relationship between SO₂ and BA accumulation is very limited. Existing studies explore only total SO₂ (TSO₂) as a variable, frequently just peripherally (Table 5).

TSO₂ had an inhibitory effect on LAB when high enough in concentration (Marcobal et al. 2006) and a low TSO₂ equated to the highest BA levels in red wines (Vidal-Carou et al. 1990a). However, to the author's knowledge, studies have not examined what impact different SO₂ addition rates (including zero addition) and their timing have on BA levels in wine.

Wells and Osborne (2012) demonstrated that LAB, particularly species such as *L. hilgardii* and *P. parvulus* (the most prolific BA producers) are potently inhibited by modest amounts of bound SO₂ (as little as 5 mg/L). It follows that use (or not) of SO₂, as well as the timing of SO₂ additions during vinification will have a significant effect on BA concentration in the resulting wine.

Since decarboxylase-positive microbial activity can be present from grapes through to finished wine, SO₂ added before AF would logically greatly reduce decarboxylase activity. This suggests that even if AA precursors were available, BA accumulation would be minimal. If no pre-AF addition is made there is an extended unprotected period for decarboxylase-positive microbial growth.

This study therefore sets out to examine the relationship between BAs and the amount and timing of SO₂ additions throughout vinification.

2.3.4. Bentonite

Use of bentonite has been shown to reduce BAs through absorption (Schneyder 1983). However, there are limited studies available There are also negative implications such as removal of colour in red wines.

Paper	Key findings on SO ₂	How SO ₂ was studied	Critique
Marcobal et al. 2006	BAs did not increase after SO ₂ addition or during wine ageing, thus suggesting inhibitory effect of SO ₂ on BA-producing microbes.	SO ₂ was universally and equally applied to all samples <u>before</u> AF (100 mg/L – a very high addition) and <u>after</u> MLF (40mg/l). Therefore, no differences in SO ₂ additions between samples.	TSO ₂ in samples was not studied. No variation in SO ₂ addition rates or timing of additions. Can only conclusively state that TSO ₂ did seem to have an inhibitory effect on bacteria. Initial addition rate is very high and would be enough to eliminate most wine bacteria from the outset.
Vidal-Carou et al. 1990a	Lower TSO ₂ range (<50 mg/L) equated to the highest BA content for red wines. No statistical difference found in white and rosé wines.	Only three limited ranges were given for TSO ₂ levels (<50, 50-150, >150 mg/L). Only limited technical information was provided (VA and lactic/malic ratios) and no other wine parameters studied.	No specific TSO ₂ values were given, and no information on amounts or timing of SO ₂ additions.
Vidal-Carou et al. 1990b	Inconclusive results regarding SO ₂ and BA level – did not find a relationship between histamine and TSO ₂ , and a non-statistical correlation was seen with tyramine.	TSO ₂ was not a variable in the study, it was only a parameter analysed in each sample (<i>n</i> =5) throughout the vinification (along with other technical analyses and BAs). All wines had TSO ₂ present from the first analysis (grapes).	A very limited sample set (<i>n</i> =5). TSO ₂ was not studied as it was considered only on an analytical level, rather than a design that incorporated addition rates and/or timing of additions. No significant or detailed statistical analysis took place.
Gardini et al. 2005	SO ₂ and BA accumulation was strongly dependent on co-factors studied such as pH, arabinose, pyridoxal 5-phosphate and ethanol concentration.	Found a complex relationship with SO ₂ and other variables studied. Only tyramine, spermidine and spermine BAs were studied.	A study very limited in scope (only one strain of <i>O. oeni</i>) in a model system (i.e. added to a sterilised media), thus not applicable to a practical level in the cellar where a vast number of microbiology and other parameters exist. TSO ₂ was only one factor in a large matrix of variables.
Ancín-Azpilicueta et al. 2016	Mixing low concentrations of SO ₂ with lysozyme and DMDC reduced BAs. The control (SO ₂ only) had a higher total amine concentration (BAs and volatile amines). Both lysozyme by itself and lysozyme mixed with SO ₂ reduced the formation of BAs but given the antioxidant activity of SO ₂ , the use of the preservative mixture seems more advisable.	7 vinifications were carried out with different treatments: lysozyme, DMDC, mixtures of lysozyme and SO ₂ and DMDC and SO ₂ . SO ₂ was used in two concentrations (25 and 50 mg/l). Results were compared with a control (50 mg/l SO ₂ only). Low BA levels were recorded across all treatments.	Not relevant to this study and an inadequate design as the wines vinified did not go through MLF, with the final sample taken immediately after AF. Low BAs are therefore to be expected. The impact of treatments beyond AF (where majority of BA accumulation happens) is therefore not known.

Table 5 - Literature including or mentioning SO₂ as a variable in BAs and wine-focused studies

2.3.5. BAs and wine style

Studies generally show that red wines have higher BA concentrations than white wines (Table 6). This is frequently attributed to red wine's almost universal completion of MLF and the presence of LAB. The literature states that because white wines in general contain fewer AA precursors, have lower pH due to the absence of skin contact during fermentation, and generally do not go through MLF, lower BA levels should be expected (Zee et al. 1983). However, Zee noted that where white and rosé wines did undergo MLF, the BA levels were close to red wine values after MLF. This can be attributed to the enhanced growth of LAB resulting from the favourable conditions created to encourage completion of MLF, rather than MLF itself.

The literature very rarely considers white wine that has undergone MLF or skin contact, even though these techniques are increasingly prevalent in white wine production, especially in premium categories. Another reason for white wine's lower BA levels in the literature could be the fact that white wine typically has both a higher TSO₂ and greater SO₂ additions at juice/must stage compared with red wine.

The absence of knowledge in this area has motivated this study's consideration of a single white variety, Sauvignon Blanc, which has undergone a wide array of winemaking practices and SO₂ regimes, including zero- and low-addition styles, to determine the impact these variables have on BA levels.

BA levels found in wine (mg/L)	Sparkling			White			Red		
	Histamine	Tyramine	Putrescine	Histamine	Tyramine	Putrescine	Histamine	Tyramine	Putrescine
Mayer and Pause, 1973				0.7 M	5.0 M	0.7 M	5.1 M	4.8 M	2.3 M
Zee et al. 1983				0.87–4.35 M	0–6.54 M	1–2.38 M	1.24–8.14 M	0–8.64 M	0–7.63 M
Zee et al. 1983 (Champagne)	10.78 M	13.70 M	6.83 M						
Zee et al. 1983 (Burgundy Pinot Noir)							9.74 M	7.31 M	9.26 M
Cillers and van Wyck 1985				0–1 R	0–2.1 R		0–49.1 R	0–6.4 R	
Baucom et al. 1986	0.5–1 R	0–0.7 R	2–3 R	0.2–9.9 R	0–0.5 R	0.7–11.7 R	1.1–11.1 R	0.0–0.2 R	0.6–5.5 R
Lehtonen et al. 1992				ND	0.5–2.8 R	1.6–2.1 R	0–15.1 R	1.2–12.8 R	3–72 R
Glória et al. 1998							2.71–7.2 M	0.71–1.62 M	10.6–27.5 M
Vazquez-Lasa et al. 1998				0.84 M	0.89 M	3.01 M	5.82–8.72 M	4–5.98 M	31.35–36.10 M
Soleas et al. 1999									
Romero et al. 2002				1.17 M	0.48 M	4.31 M	2.75 M	2.91 M	9.59 M
Kállay and Sardy, 2003				0–23.7 R	2.1–6.9 R	2.7–30 R	0–4.1 R	0.5–7.8 R	2.3–15.7 R
Bover-Cid et al. 2006				ND–1.1 R	ND–2.3 R	2.1–9.7 R	ND–19.6 R	ND–18.2 R	3.7–99.9 R
Soufleros et al. 2007				ND–5.95 R	ND–2.54 R	ND–3.22 R	ND–2.11 R	ND–3.65 R	ND–5.23 R
Konakovsky et al. 2010							0.5–26.9 R	1.1–10.7 R	2.9–122 R
EFSA 2011				0.8–0.9 R	1.1–1.2 R	1.4–1.5 R	3.6–3.7 R	2.7–2.9 R	4.2–4.8 R
Henríquez-Aedo et al. 2012				0.51–2.91 R	ND–0.42 R	3.37–12.86 R	1.12–15.85 R	0.37–6.31 R	5.76–34.4 R
Martuscelli et al. 2013				0–3.4 R	0–6.8 R	0.8–12.8 R	0–10.8 R	0–18.8 R	2.4–31.8 R
Tuberoso et al. 2014				ND	NQ	1.48–10.55 R	0–8.11 R	5.08–11.5 R	11.4–32.8 R
Esposito et al. 2019				0.76 M	0.38 M	1.85 M	2.36 M	3.43 M	9.98 M

M = mean, R = range

Table 6 - BA levels in mg/L analysed in commercially finished, bottled red, white and sparkling wines

3. RESEARCH QUESTIONS

RQ1 - What is the relationship between the amount of SO₂ added during winemaking and the resulting levels of BAs in wine?

RQ2 - What is the relationship between the timing of SO₂ additions during winemaking and the resulting levels of BAs in wine?

RQ3 - Is there an argument for the creation of a new low-BA category of wine to aid those who suffer negative side effects from BAs, empowering them to make more confident wine-purchasing decisions?

4. METHODOLOGY

4.1. Wine samples

One hundred New Zealand Sauvignon Blanc wine samples were purchased from commercial sources or supplied by the wine producer. All wines had been bottled within the four years prior to this study's analyses. Samples were commercial still wines (750 mL bottles), to reflect real consumer exposure to BAs. Vintages were predominantly 2019 ($n=47$) and 2018 ($n=27$), with a small number of 2017 ($n=14$), 2016 ($n=8$) and 2015 ($n=4$). Wines selected ensured a proportionate cross-section of SO₂ regimes and wine styles. A technical data template was sent to every wine producer to obtain requisite information for each sample (Appendix 4).

Two sample groups were created using the technical information obtained: sample group 1 (SG1) ($n=100$) categorised samples into four subgroups by SO₂ regime/style to address RQ1 (Table 7), and sample group 2 (SG2) ($n=98$) categorised samples into five subgroups by when the first SO₂ addition was made in order to address RQ2 (Table 8).

SG1 subgroups	Winemaking techniques used	SO ₂ regime / TSO ₂	Number of samples (n)
Zero-addition	No selection based on winemaking techniques	No SO ₂ additions made throughout vinification	10
Low-addition	No selection based on winemaking techniques	≤ 40 mg/L TSO ₂	11
Alternative	Selected if two or more of following criteria met: full/partial MLF, lees contact (>3 months), lees stirring, oak fermentation and/or maturation	>40 mg/L TSO ₂	48
Classic	Selected if all following criteria met: 0–10% MLF, stainless steel AF (>50%), ≤ 3 months lees contact	Total SO ₂ additions >65 mg/L or TSO ₂ >80 mg/L	31
TOTAL			100

Table 7 – Sample group 1 (SG1) categorising samples studied by SO₂ regime and wine style (n=100)

SG2 subgroups	Stage of vinification that sample received first SO₂ addition	Number of samples (n)
Stage 1 (S1)	Pre-AF	60
Stage 2 (S2)	Post-AF	7
Stage 3 (S3)	Post-MLF / post-assemblage	10
Stage 4 (S4)	Pre-bottling	11
Zero-addition	No SO ₂ addition at any stage	10
TOTAL		98*

*Two outliers were removed from this sample group – see section 5.2.5.

Table 8 – Sample group 2 (SG2) categorising samples by when first SO₂ addition was made (n=98)

4.2. Laboratory analysis

Three BAs – histamine, tyramine and putrescine – were selected for analysis using reverse-phase (RP) high-pressure liquid chromatography (HPLC)¹⁰ using an Agilent Technologies Agilent 1200 Series instrument (Waldbronn, Germany). HPLC was performed according to an adapted method from Kelly et al. (2010). The protocol is described in detail in Appendix 5.

To prepare each sample for analysis, 1000 µl of wine sample and 1000 µl of ultrapure water was vortexed and syringe filtered (0.45 µm filter) into an individual HPLC vial. Unfiltered wines were centrifuged prior to sample preparation.

The vials were numbered and placed into the HPLC sequence programme with 10–12 samples completed each day. Analyses were performed in duplicate and in randomised order.

Chromatographic data were collected and analysed with an OptiPlex 7070 system (Dell). Results were expressed as mean values in milligrams per litre of wine.

¹⁰ HPLC is the most widely recognised method for BA analysis (Ruiz-Capillas and Herrero 2019).

4.2.1. Method validation

The sample analytes were identified by comparison with the retention times of the mixed control standard. Quantification was performed by the external standard method based on the peak areas measured by the data system.

Quantification limits were applied from Kelly et al. (2010). Limit of Quantification (LOQ) for histamine, tyramine and putrescine was calculated as 0.25 mg/L. The lowest concentrations were recorded for tyramine (LOQ $n=9$ and ND $n=2$). Putrescine and histamine concentrations for all samples were >LOQ.

4.3. Statistical methods

All statistical analyses were performed in open-source statistical platform R using in-house R scripts (<https://www.r-project.org/>).

No missing value substitution was required. The data were not normally distributed; thus log-transformation was performed prior to further analysis (Appendix 6). One-way analysis of variance (ANOVA) was performed for SG1 and SG2 with the subgroups as the source of variation. If significance was found, a post-hoc Fisher Least Significant Difference (LSD) test was utilized for separation of means (Appendix 7). These results demonstrated whether there were significant differences in BA concentrations among the subgroups.

Boxplots were used to graphically summarise i) the total and individual BA concentrations and ii) TSO₂, FSO₂ and total SO₂ additions. Principal component analysis (PCA) was used to graphically demonstrate the subgroups' clustering based on the statistical variation in the data. Pearson's correlation coefficient analysis was performed to determine the direction and strength of the correlations between BA concentrations and SO₂ parameters. Lastly, heatmaps were produced to graphically

show the relative abundancies of variables in both SG1 subgroups and individual samples.

Table 9 summarises the statistical methods utilised for RQ1 and RQ2.

Research Question (RQ)	Statistical method	What is demonstrated
RQ1	One-way ANOVA with post-hoc Fisher's LSD	Statistical relevancies between groups in SG1
	Boxplots	Normalised concentration of BA and SO ₂ variables for each group in SG1
	Heatmaps – group average and detail view	Relationship between concentration of BAs, SO ₂ variables and pH across SG1
	Principal component analysis (PCA) scores plot	Clustering of groups in SG1 according to BA and SO ₂ variables
	Pearson's correlation coefficient	Correlation relationship between BAs and SO ₂ variables.
RQ2	One-way ANOVA with post-hoc Fisher's LSD	Statistical relevancies between groups in SG2
	Boxplots	Normalised concentration of BA for each SO ₂ addition stage in SG2
	Principal component analysis (PCA) scores plot	Clustering of groups in SG2 according to BA and SO ₂ timing variables
	Pearson's correlation coefficient	Correlation relationship between BAs and SO ₂ first-addition timing variables. Relationship between MLF and BAs in MLF sample group (MLFSG)
	Student's t-test for paired samples and fold-change values	Relevance of whether a S1 addition affects BA concentration in MLFSG sub-groups

Table 9 - Statistical methods used for analyses of RQ1 and RQ2

5. RESULTS AND CONCLUSIONS

Each research question is addressed successively by analysis of results and discussion.

5.1. RQ1: Relationship between BAs and amount of SO₂ added

5.1.1. BA, SO₂, and pH analyses for SG1

Table 10 presents the analyses of BAs, SO₂ concentrations and pH for SG1. Values in mg/L are presented as mean ± standard deviation, with statistical relevancies shown. Where statistical difference was observed, the *p*-values (FDR adjusted) were all highly significant (*p* <0.05) (ANOVA table: Appendix 7).

		Zero-addition	Low-addition	Alternative	Classic
Biogenic amines	Total BAs	37.21 ± 20.86	18.91 ± 14.78	7.52 ± 3.57	4.59 ± 1.75
	Histamine	11.22 ± 6.75 A	6.54 ± 4.38 A	3.36 ± 1.79	2.01 ± 1
	Tyramine	5.64 ± 4.21 A	2.05 ± 1.75 A	0.60 ± 0.30 F	0.39 ± 0.24 F
	Putrescine	20.35 ± 11.71	10.32 ± 9.56	3.58 ± 2.41	2.27 ± 1.03
SO₂ concentrations and stage-wise additions	Total SO ₂	5 ± 3	24 ± 9	95 ± 27 F	112 ± 26 F
	Free SO ₂	2 ± 1	14 ± 12	27 ± 9 F	30 ± 5 F
	1. Pre-AF addition	0 A	0 A	24 ± 25	44 ± 22
	2. Post-AF addition	0 A	1 ± 4 A	32 ± 27	48 ± 21
	3. Post-MLF / post-assemblage addition	0 AC	3 ± 10 AE	21 ± 27	3 ± 10 CE
	4. Pre-bottling addition	0	25 ± 8	25 ± 17 F	22 ± 16 F
pH	Total SO ₂ addition	0	29 ± 9 DE	102 ± 35 DF	117 ± 33 FE
	pH	3.55 ± 0.17	3.47 ± 0.21	3.21 ± 0.15 F	3.21 ± 0.13 F

Values (mg/L) are means ± standard deviation. Values not sharing the same capital letter within each row are significantly different at *p* <0.05.

Table 10 – Analyses of BAs, SO₂ (mg/L) and pH across SG1 subgroups
(n=100)

Boxplots demonstrate key patterns between SG1 subgroups (emboldened for clarity) and BA concentrations (Figure 3). **Zero-addition**¹¹ and **low-addition**¹² had the highest concentrations across all BA variables, with **zero-addition** recording the three highest total BA (TBA) concentrations¹³ (70.8, 59.6 and 58.25 mg/L respectively).

Conversely, **classic**¹⁴ had the lowest BA concentration. However, there was less statistical difference in BAs observed between **classic** and **alternative**¹⁵ and no statistical difference between them for tyramine. **Zero-addition** and **low-addition** were not statistically different from each other in histamine or tyramine but were highly statistically different in putrescine and TBA.

95th percentile analyses make this linear relationship even more apparent, with a clear increase in BAs from **classic**—**zero-addition** (Table 11).

	Zero-addition	Low-addition	Alternative	Classic
Histamine	20.64	15	6.54	3.65
Tyramine	12.05	4.89	0.96	0.66
Putrescine	38.61	29.55	8.63	3.94
Total BAs	65.2	48.48	15.02	7.3

Table 11 - 95th percentile concentrations of BAs (mg/L) across SG1 subgroups (n=100)

Every group was highly statistically different for TBA and putrescine concentrations, demonstrating there are clear differences in BA levels among the four subgroups.

¹¹ As defined in Table 7, zero-addition wines did not need to satisfy any specific winemaking criteria except for having no SO₂ additions throughout vinification.

¹² As defined in Table 7, low-addition wines did not need to satisfy any specific winemaking criteria except for having ≤40 mg/L TSO₂.

¹³ Total BA concentration in this context is the sum of histamine, tyramine and putrescine.

¹⁴ As defined in Table 7, classic wines must have met all of the following criteria: 0–10% MLF, stainless steel AF (>50%), ≤3 months lees contact, and total SO₂ additions >65 mg/L or >80 mg/L TSO₂.

¹⁵ As defined in Table 7, alternative wines must have met two or more of following criteria: full/partial MLF, lees contact (>3 months), lees stirring, oak fermentation and/or maturation and >40 mg/L TSO₂.

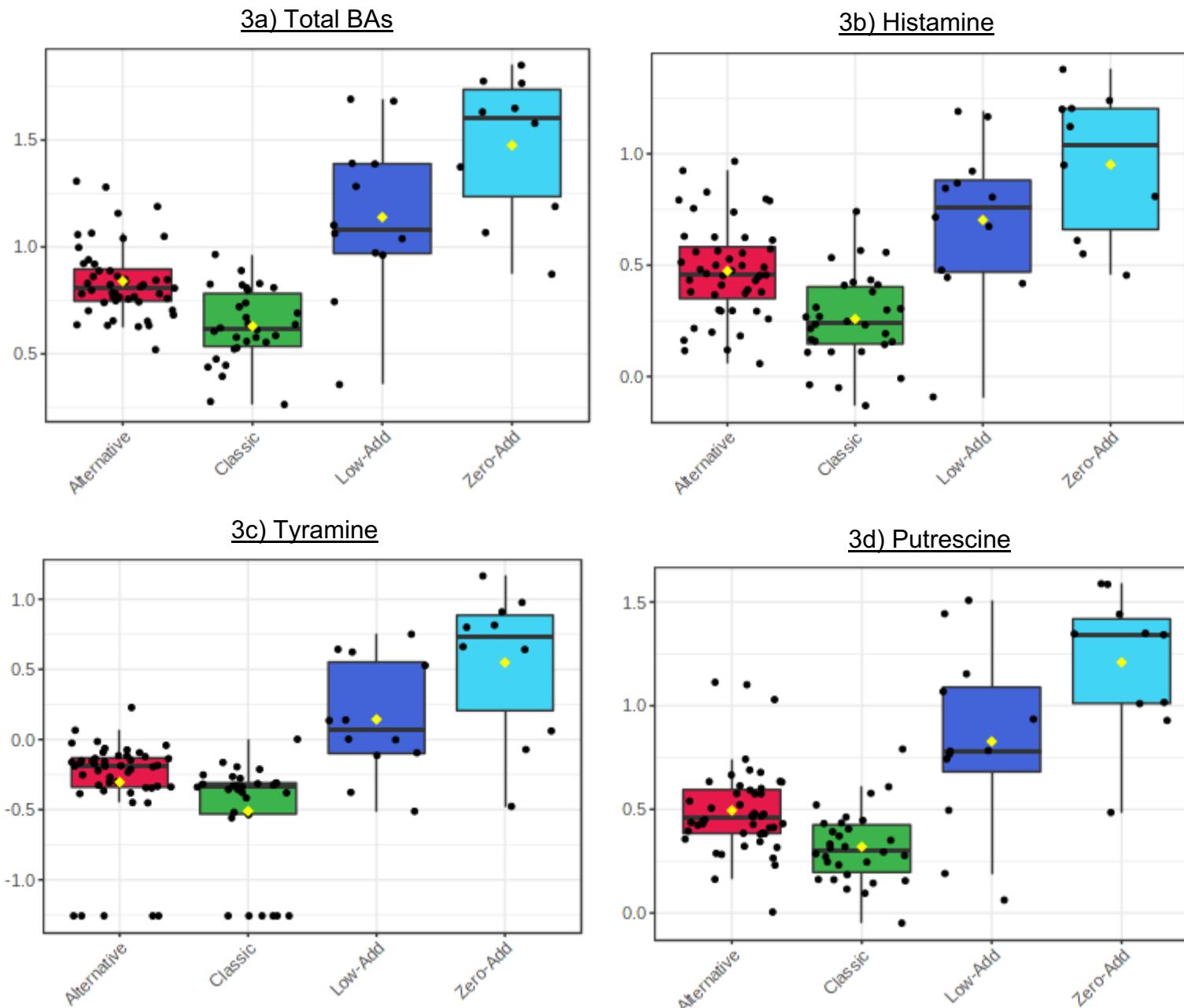


Figure 3 - Boxplots showing normalised concentration of (a) total BAs, (b) histamine, (c) tyramine and (d) putrescine across SG1 ($n=100$)
Y-axis represents normalised data

Boxplots show the SO₂ concentrations across SG1 (Figure 4). As expected, SO₂ concentrations increased from **zero-addition – low-addition – alternative – classic** across all SO₂ variables, except for post-MLF addition in **classic** because MLF was not carried out in this category. **Zero-addition** had the highest average pH (pH 3.55) followed by low-addition (pH 3.47), while **alternative** and **classic** were identical (pH 3.21). The differences in pH can be attributed to several **zero-addition** and **low-addition** samples with very high pH. A common variable between them was significant periods of time on skins (>7 days), which would have increased pH.¹⁶ Processing techniques such as foot-treading and basket-pressing could also increase pH.¹⁷

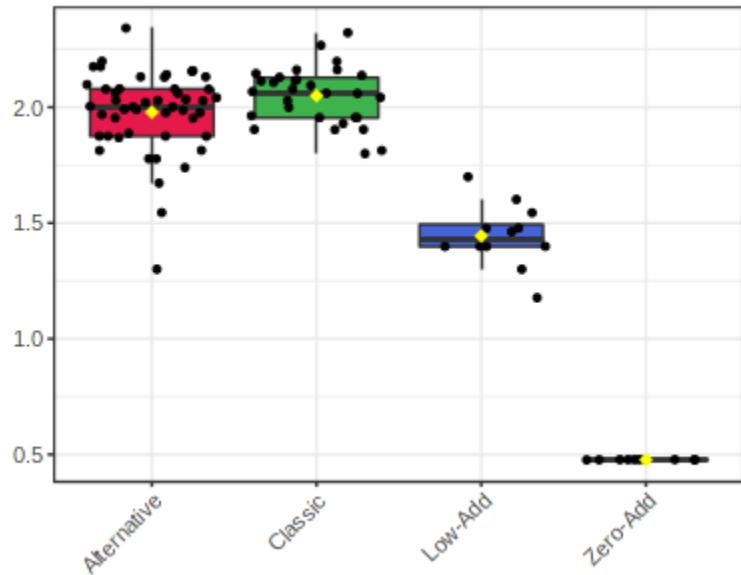
Furthermore, the absence of SO₂ in **zero-addition** wines leads to a high probability that all samples had gone through MLF, despite what the producer believed or had disclosed, which would account for the higher average pH in this category.

The differences in SO₂ regime between SG1 subgroups are relevant to style and are accordingly included in Table 10, but timing of SO₂ addition(s) is discussed in RQ2, section 5.2.

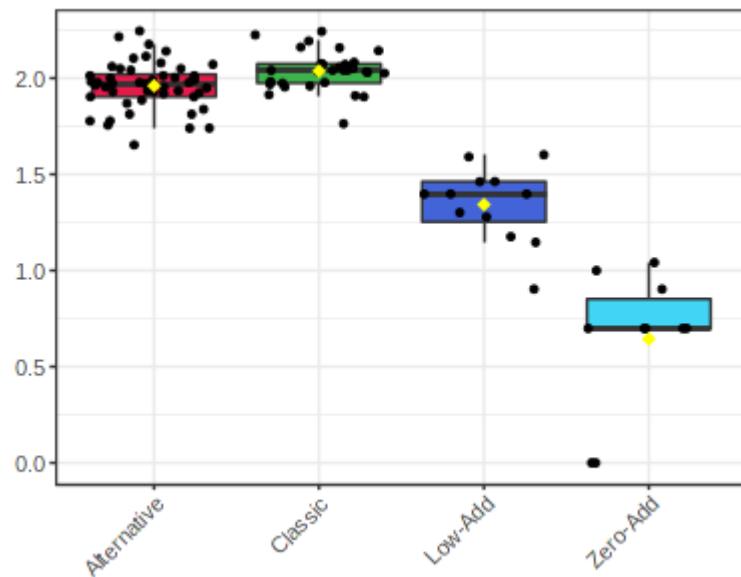
¹⁶ This is due to the increased extraction of potassium from the grape skins, which increases juice pH (Australian Wine Research Institute (AWRI) 2020c).

¹⁷ Both practices increase skin contact, while basket pressing also can result in increased pressure and therefore greater extraction. Although this technical information was not obtained, both these categories of wine are likely to undergo processing techniques such as this.

4a) Total SO₂ additions



4b) Total SO₂



4c) Free SO₂

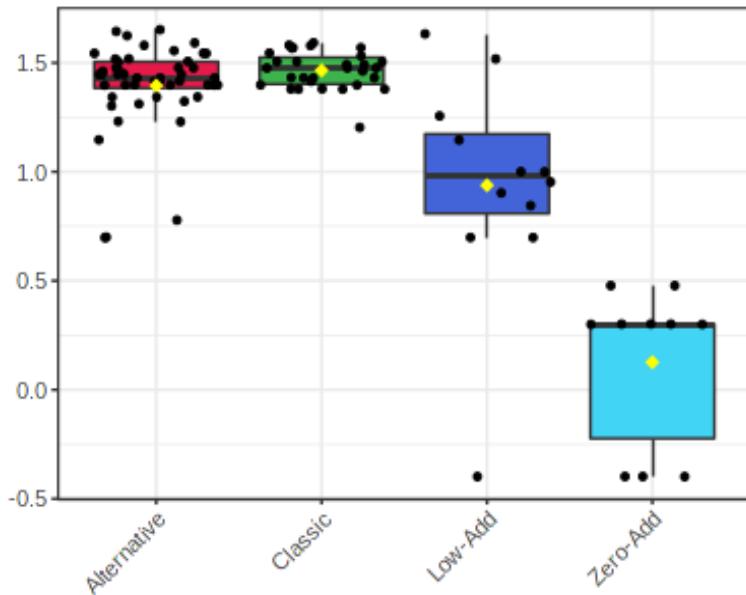


Figure 4 - Boxplots showing (a) total SO₂ additions, (b) total SO₂ and (c) free SO₂ across SG1 subgroups (n=100)
Y-axis represents normalised data

Principal component analysis (PCA) demonstrates the clear clustering patterns among SG1 subgroups. BAs are the main contributors behind this clustering (Figure 5).

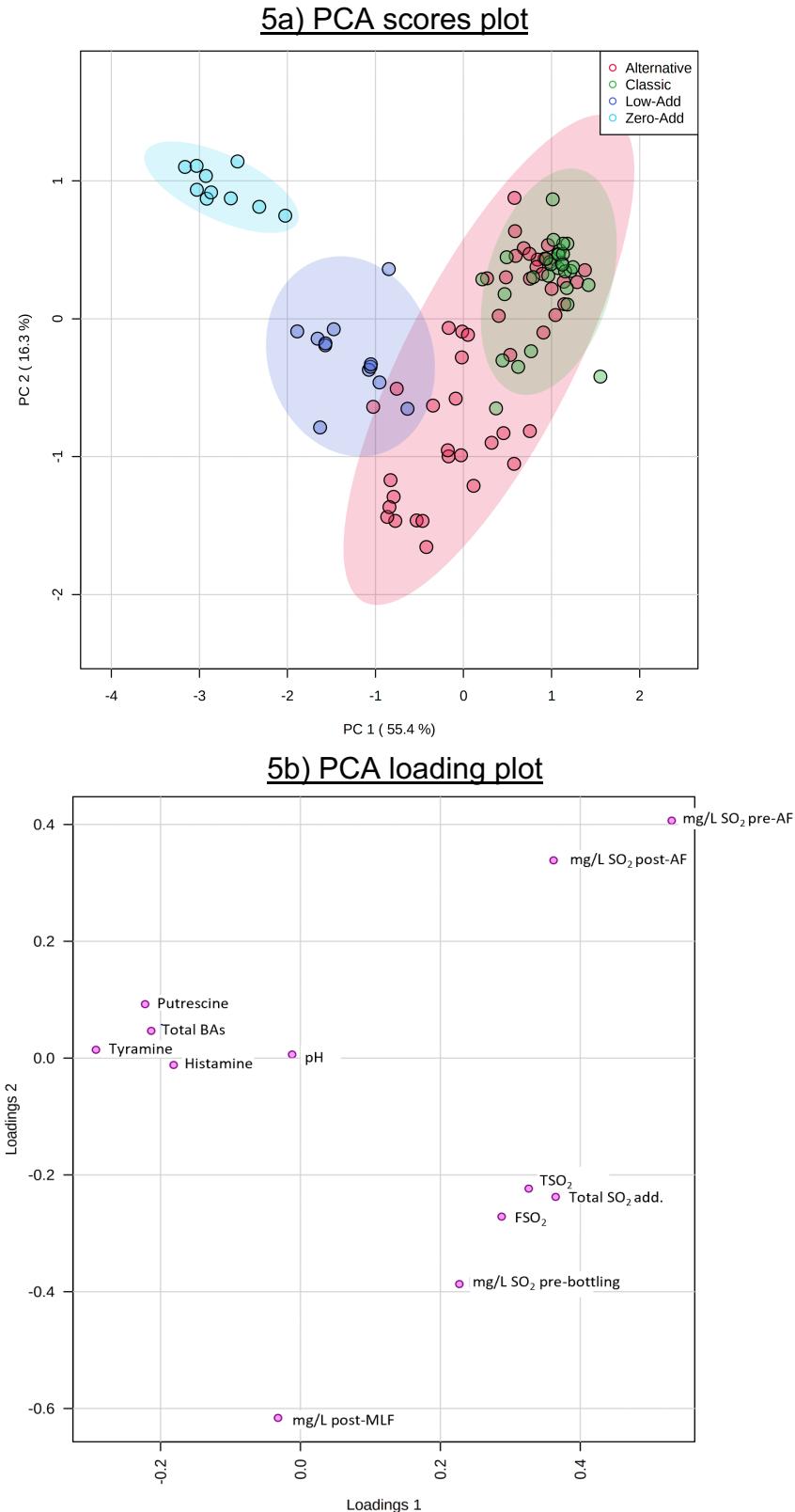
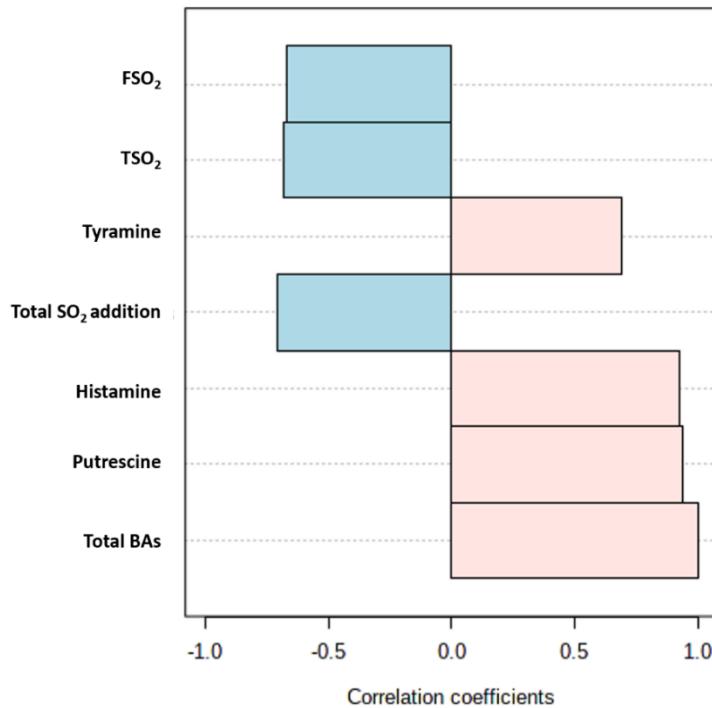


Figure 5 – Two-dimensional principal component analysis (PCA) (a) scores plot and (b) loading plot showing clustering patterns and significant variables for SG1 subgroups ($n=100$)

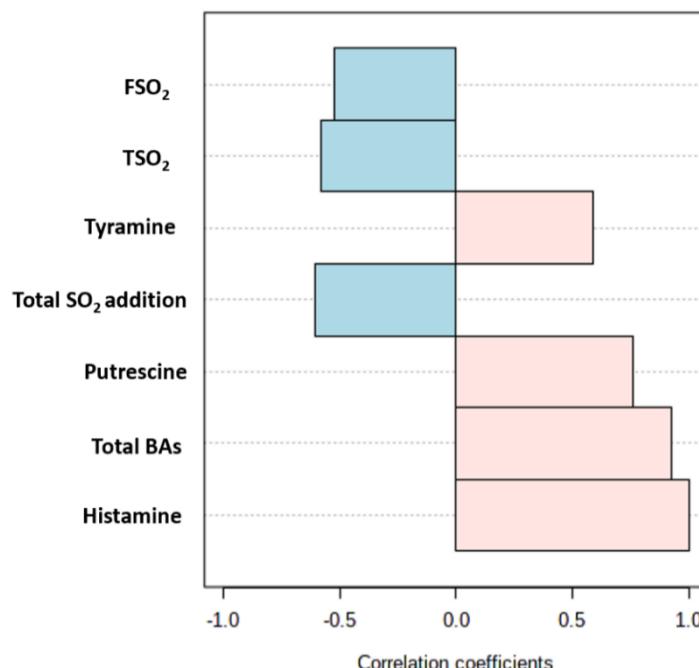
5.1.2. Correlation between BAs and SO₂

Correlation coefficient analyses confirm the negative correlation between BAs and total SO₂ addition, TSO₂ and FSO₂ (Charts: Figure 6, Table: Appendix 7). BAs have a positive correlation with each other and with pH values.

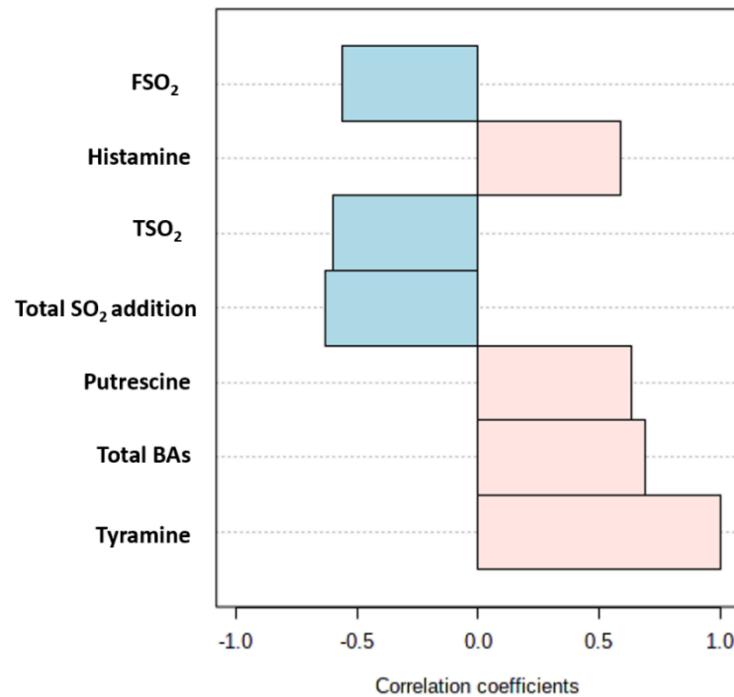
6a) Correlation of total BAs with BAs and SO₂



6b) Correlation of histamine with BAs and SO₂



6c) Correlation of tyramine with BAs and SO₂



6d) Correlation of putrescine with BAs and SO₂

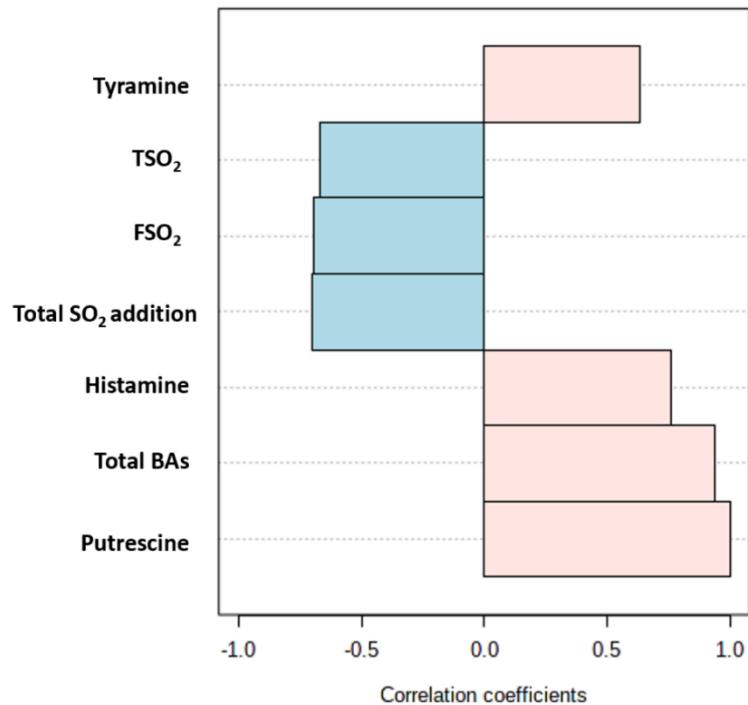


Figure 6 - Correlation coefficients for (a) total BAs, (b) histamine, (c) tyramine and (d) putrescine with BAs and SO₂ in SG1 subgroups (n=100)

Heatmaps reinforce these relationships, demonstrating that BAs and pH values contrast with SO₂ concentrations. Figure 7 shows group average concentrations, while Figure 8 shows the detail view. The relative abundances are scaled, with the negative value (blue) indicating least, and positive value (red) indicating most of the particular variable.

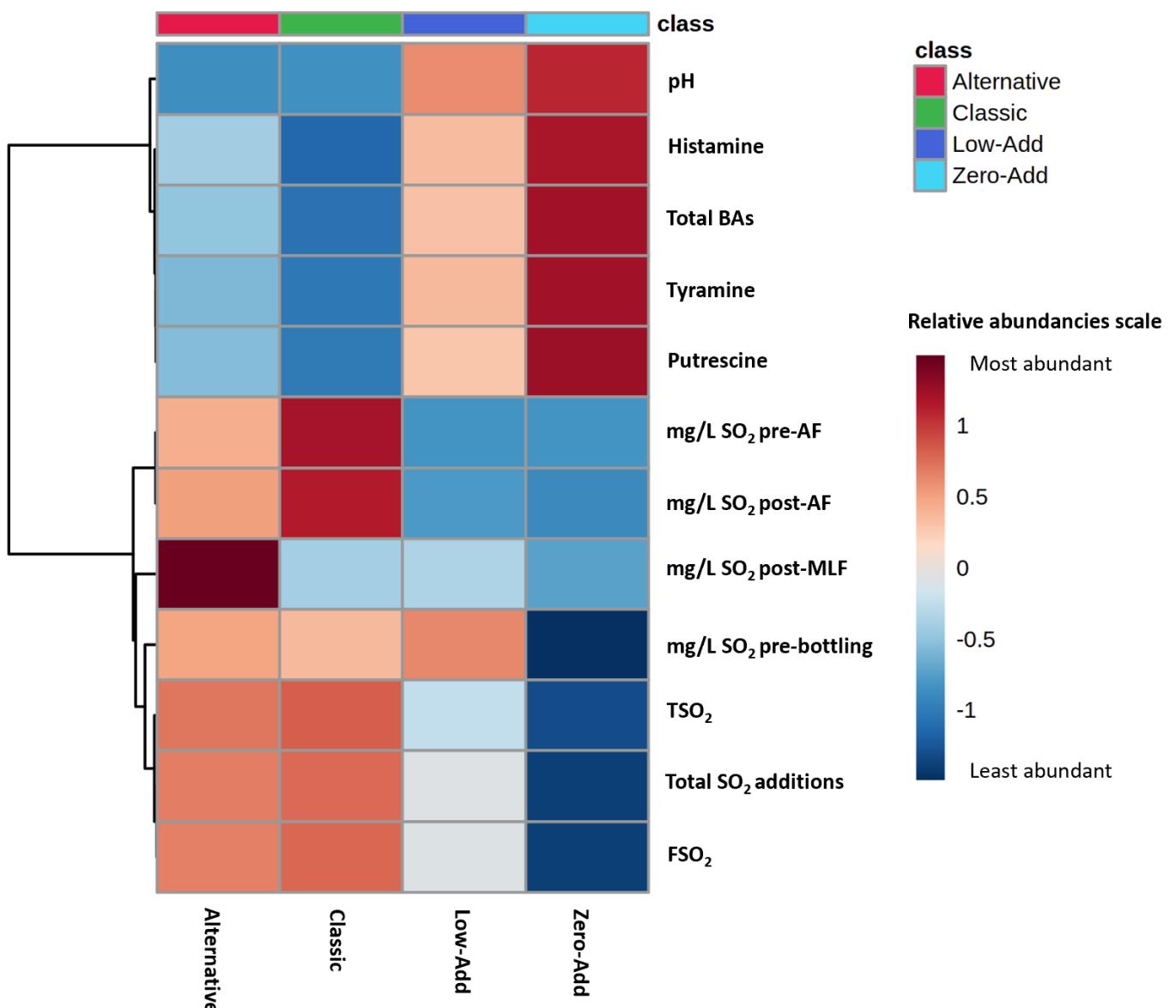


Figure 7 – Group view globally scaled heatmap of SG1 subgroups showing average BA and SO₂ concentrations and pH values (n=100)

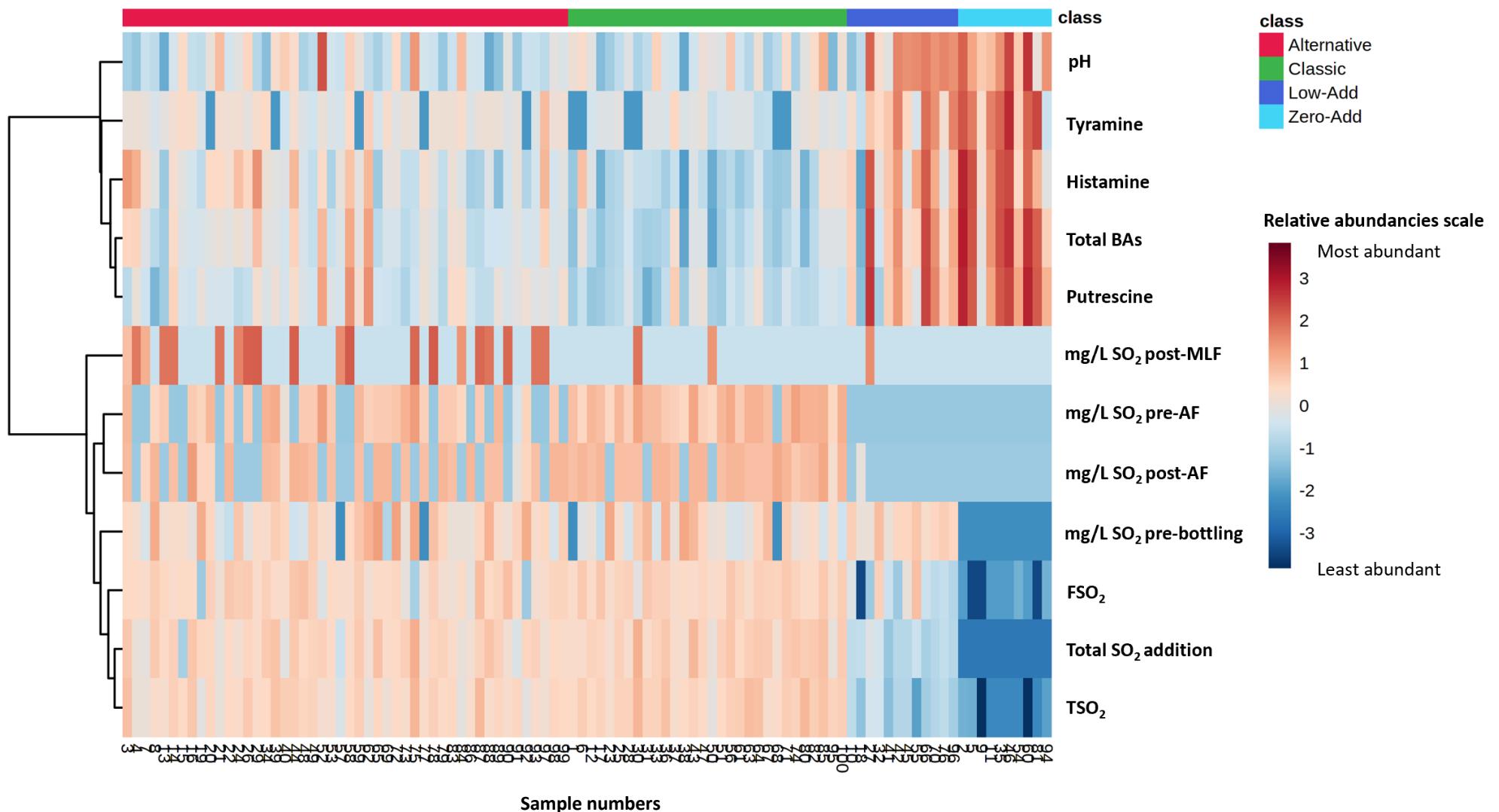


Figure 8 – Detail view of globally scaled heatmap showing relative abundancies of SO₂, BA and pH variables across all samples in SG1 (n=100)

Collectively these analyses demonstrate that BAs are highly impacted by SO₂. Total SO₂ additions and, consequently, TSO₂ and FSO₂ have a strong negative correlation with the resulting BA levels in wine. BA concentrations in wine are therefore acutely impacted by the amount of SO₂ used throughout vinification. Wines with zero- or low-TSO₂ are significantly more likely to have higher BA concentrations than wines with conventional SO₂ additions, regardless of winemaking techniques used. This can be asserted because the **alternative** subgroup displayed a wide range of winemaking techniques within it, including MLF, skin contact and lees ageing/stirring, which was not dissimilar to **zero-addition** and **low-addition** styles. SO₂ regime is the defining difference among them.

In general, higher total additions of SO₂ throughout vinification result in lower BA concentrations, independent of timing of additions.

5.2. RQ2: Relationship between BAs and timing of SO₂ additions

5.2.1. SG2: BA and pH analyses and timing of first SO₂ addition

Table 12 presents the analyses of BA concentrations and SO₂ parameters in respect of sample group 2 (SG2), grouped according to when first SO₂ addition was made.

Values in mg/L are mean ± standard deviation and statistical relevancies are shown.

Where statistical difference was observed, p-values (FDR adjusted) were highly significant ($p < 0.05$) (ANOVA table: Appendix 7).

SG2 subgroups		Stage 1 (S1) (Pre-AF)	Stage 2 (S2) (Post-AF)	Stage 3 (S3) (Post-MLF/ post- assemblage)	Stage 4 (S4) (Pre-bottling)	Zero-addition (No SO ₂ additions throughout vinification)
Biogenic amines	Total BAs	5.80 ± 2.91 A	7.26 ± 2.36 AE	13.99 ± 12.35 E	16.54 ± 11.79 J	37.21 ± 20.86 J
	Histamine	2.48 ± 1.33 A	3.27 ± 1.39 A	6.17 ± 3.67 H	5.91 ± 3.39 H	11.22 ± 6.75
	Tyramine	0.48 ± 0.26 A	0.64 ± 0.12 AE	0.86. ± 0.37 EH	2.17. ± 1.77 H	5.64 ± 4.21
	Putrescine	2.89 ± 1.96 A	3.54 ± 1.2 AE	6.95 ± 8.91 EH	8.47 ± 7.09 H	20.35 ± 11.71
SO ₂ concentration and stage-wise additions	S1 Pre-AF	42 ± 21	0 EFG	0 EHI	0 FHJ	0 GIJ
	S2 Post-AF	42 ± 24 A	52 ± 11 A	0 HI	0 HJ	0 IJ
	S3 Post-MLF / post-assemblage	6 ± 16 CD	34 ± 25	53 ± 13	0 CJ	0 DJ
	S4 Pre-bottling	25 ± 17 ABC	22 ± 13 AEG	20 ± 10 BEH	26 ± 7 CFH	0
	Total SO ₂ additions	115 ± 33 A	108 ± 31 A	73 ± 20	26 ± 7	0
	Total SO ₂	106 ± 29 A	92 ± 17 AE	76 ± 22 E	30 ± 22	5 ± 3
	Free SO ₂	28 ± 8 AB	27 ± 6 AE	26 ± 10 BE	17 ± 12	2 ± 1
pH	pH	3.21 ± 0.15	3.18 ± 0.1	3.27 ± 0.18	3.47 ± 0.19	3.55 ± 0.17

Values (mg/L) are means ± standard deviation. Values not sharing the same capital letter within each row are significantly different at $p < 0.05$.

Table 12 – Analyses of BAs, SO₂ (mg/L) and pH across SG2 subgroups (n=98)

Stage 1 (S1) had the lowest BA concentrations across all BA variables, while **zero-addition** had the highest. Boxplots present the BA concentrations across SG2 (Figure 9).

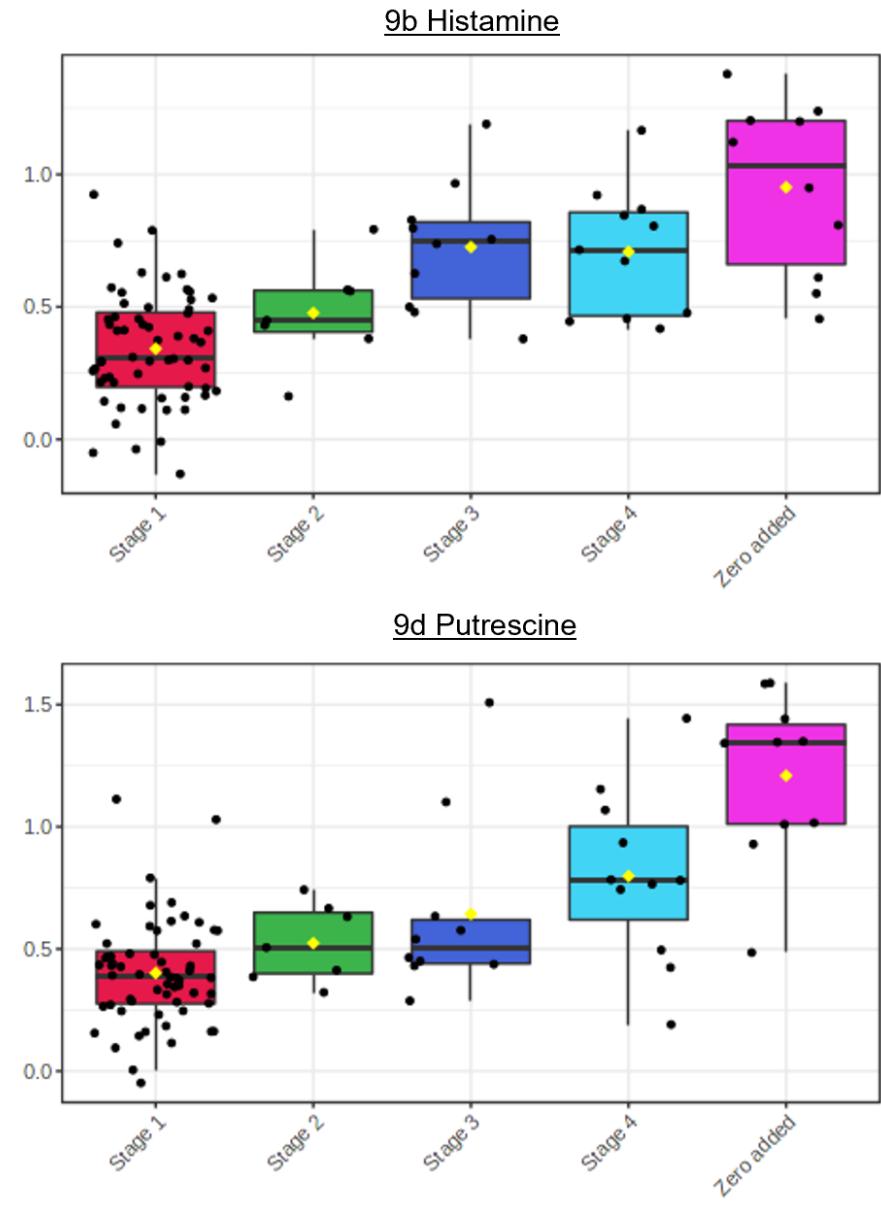
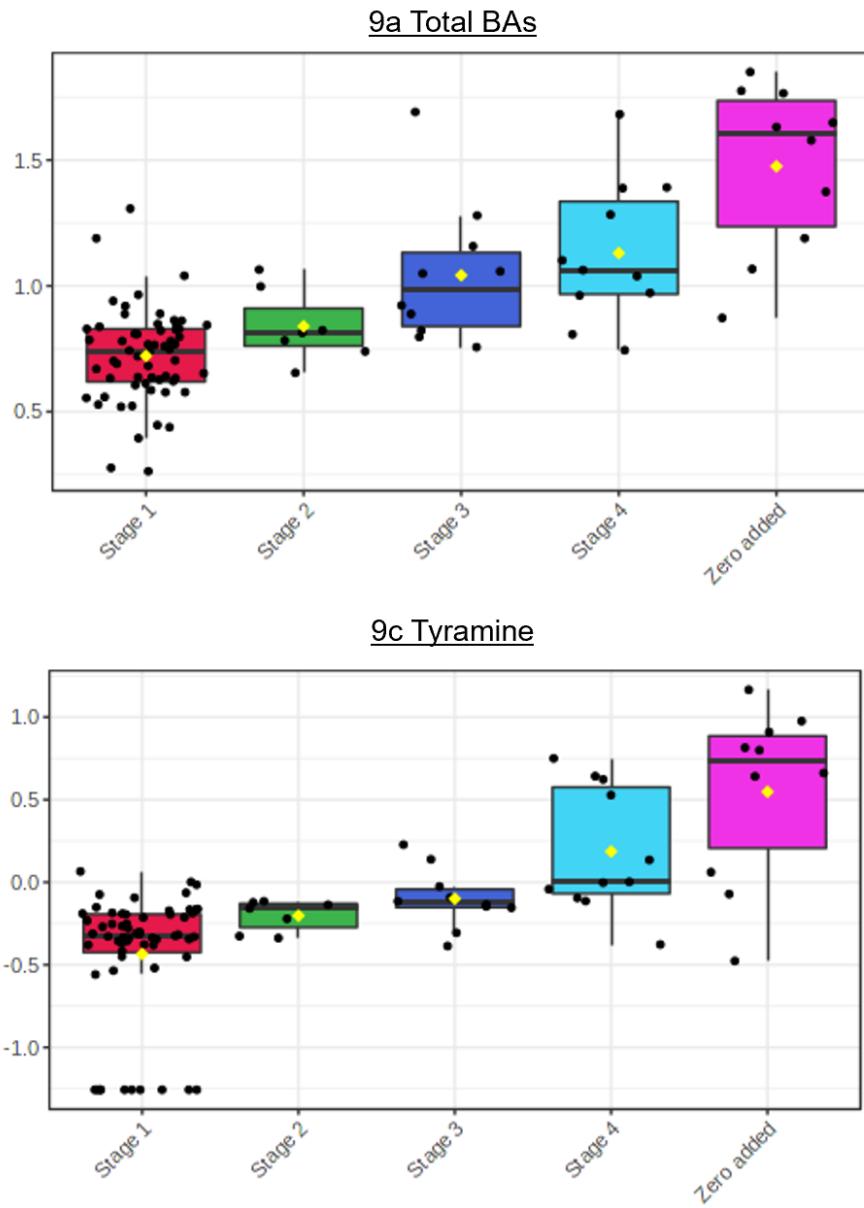


Figure 9 - Boxplots of normalised concentrations of (a) total BAs, (b) histamine, (c) tyramine, and (d) putrescine across SG2 ($n=98$)
Y-axis represents normalised data

A PCA scores plot shows the clustering between the groups (Figure 10). It clearly demonstrates **zero-addition** wines were highly divergent from the other groups.

Reviewing stages 1–4 only, correlation coefficients for each BA as well as the TBA concentration shows that in every instance, S1/pre-AF addition had the strongest negative correlation with the BA variable. The largest negative correlation for S1 addition was histamine, while putrescine and tyramine were very similar (Chart: Figure 11, Table: Appendix 7).

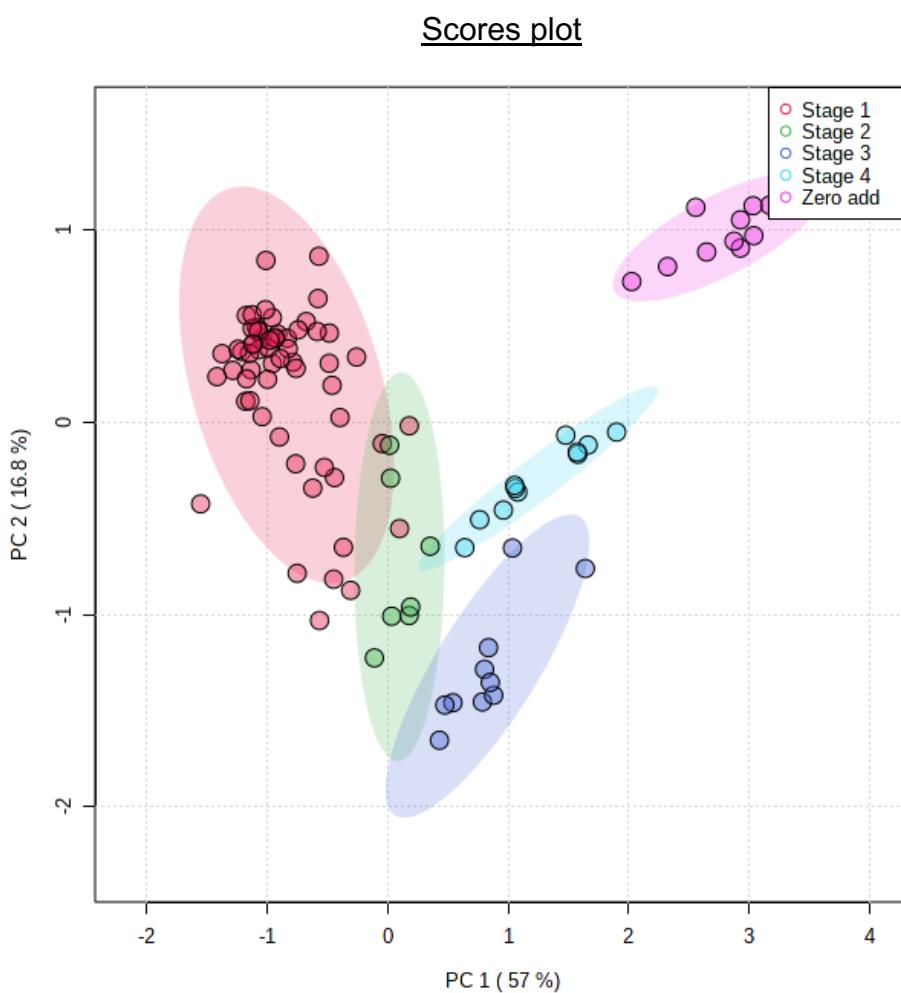
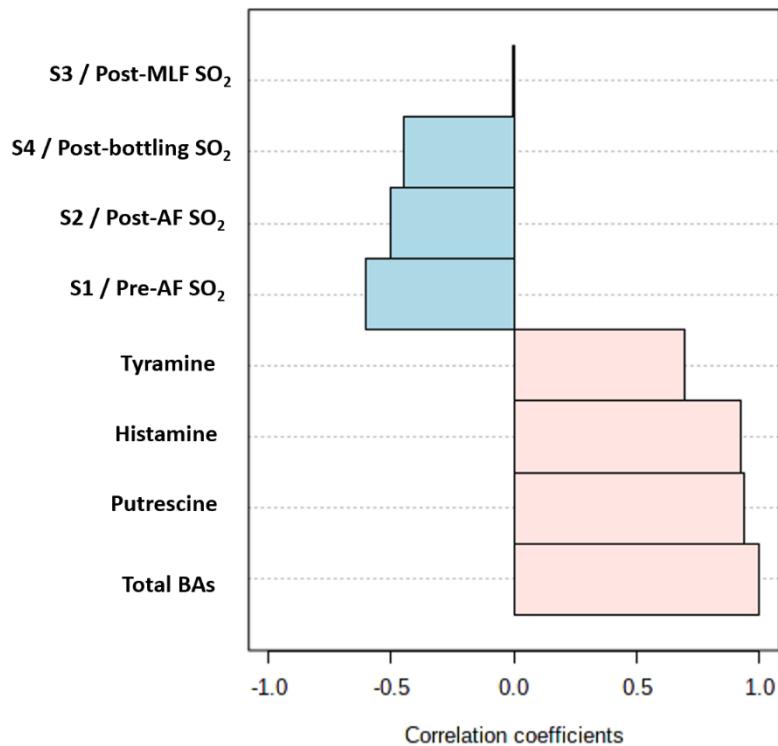
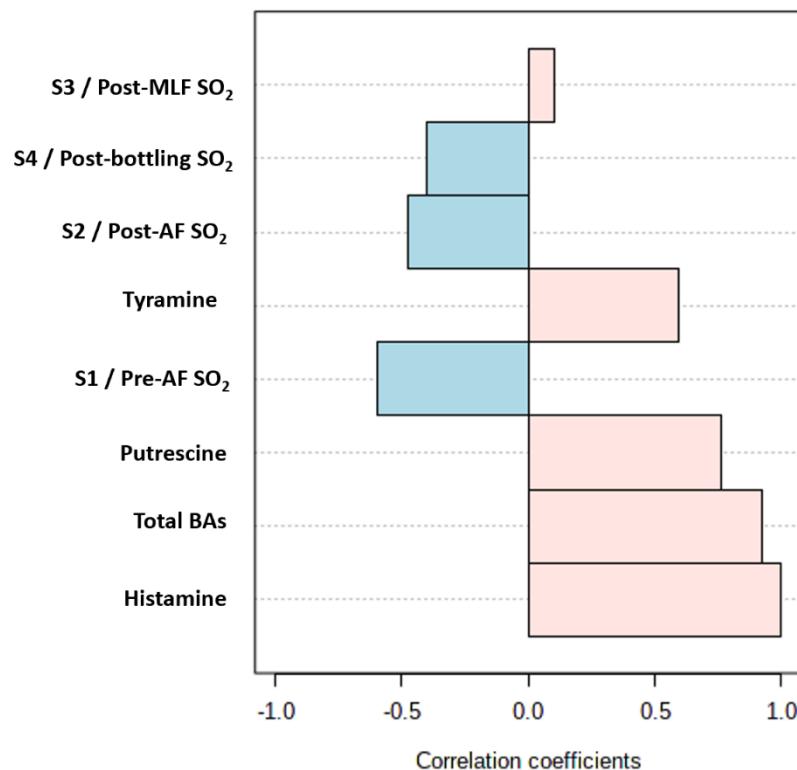


Figure 10– Two-dimensional principal component analysis (PCA) scores plot showing clustering patterns of SG2 subgroups (n=98)

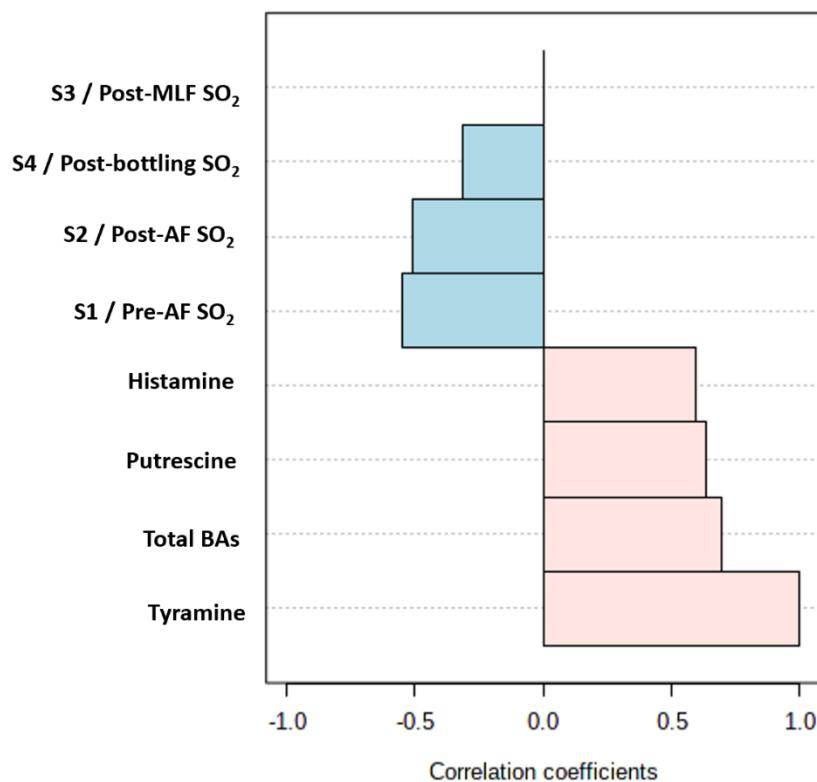
11a) Correlation of total BAs with BAs and timing of SO₂ addition



11b) Correlation of histamine with BAs and timing of SO₂ addition



11c) Correlation of tyramine with BAs and timing of SO₂ addition



11d) Correlation of putrescine with BAs and timing of SO₂ addition

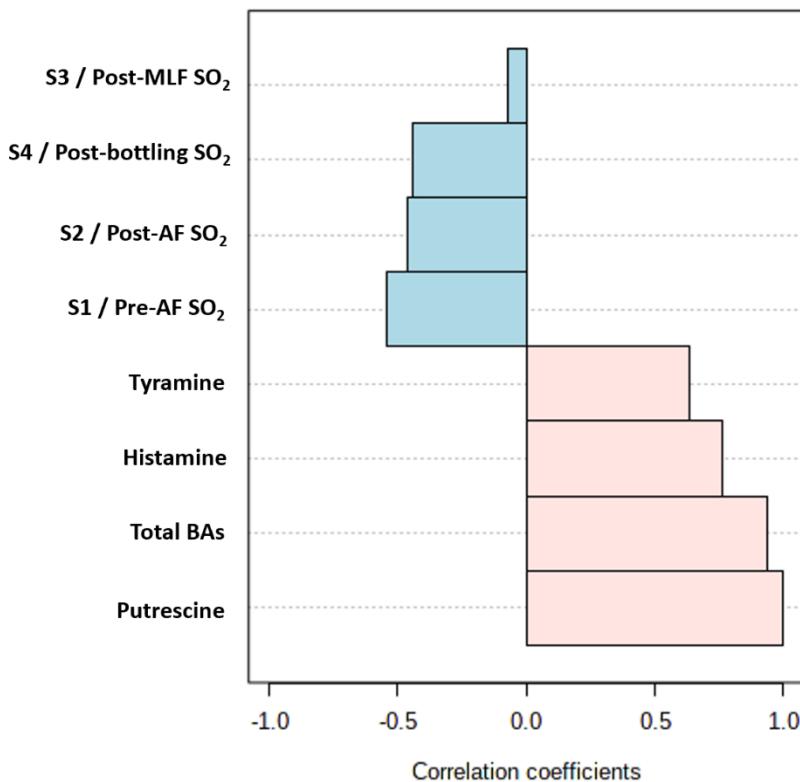


Figure 11 - Correlation coefficients of (a) total BAs, (b) histamine, (c) tyramine, and (d) putrescine with BAs and timing of SO₂ addition (S1—S4) (n=98)

Evidentially, S1 addition of SO₂ produced the lowest BA concentrations. However, S1 wines were not statistically different from S2 wines ($p > 0.05$). Therefore, it is necessary to explore these results, considering what contribution MLF has.

5.2.2. Pre-AF addition across SG2

When the dataset is split into respective MLF sample groups (MLFSG): full-, partial- and no-MLF, the BA concentrations for full-MLF samples were significantly higher than no-MLF samples (Figure 12). This result is expected, as conditions required for MLF are hospitable for bacterial growth, thereby promoting decarboxylase-positive bacteria.

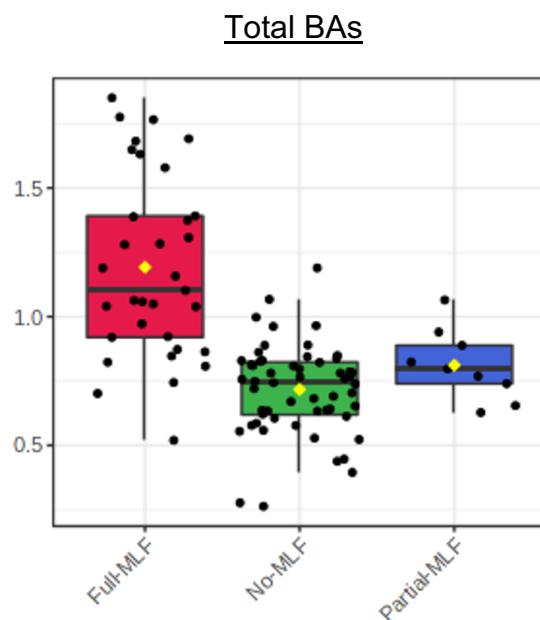


Figure 12 – Boxplot of total BA concentration across MLFSG subgroups (n=98)
Y-axis represents normalised data

Correlation coefficient analyses demonstrates this positive relationship between MLF and BA production (Figure 13).

Correlation of MLF with BA, SO₂ concentrations and pH

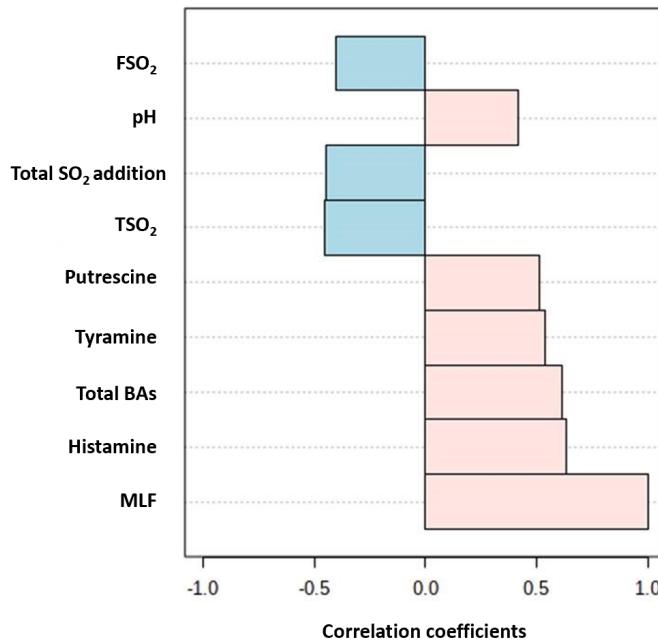


Figure 13 - Correlation coefficients of MLF with BA and SO₂ concentrations and pH (n=100)

Table 13 shows the number of wines that went through full or partial MLF (100% spontaneous in all cases) across SG2. There are no full-MLF samples in S2 because a post-AF SO₂ addition will result in FSO₂, which is toxic to MLF-performing LAB. The four wines in S2 that underwent partial MLF (5–50%) received an SO₂ addition to the non-MLF portion.

	Full-MLF	Partial-MLF
S1 Pre-AF addition	8	4
S2 Post-AF addition		4
S3 Post-MLF addition	7	2
S4 Pre-bottling addition	10	
Zero-addition	8	
Total	33	10

MLF is 100% spontaneous in every case

Table 13 – Number of samples that went through full MLF or partial MLF across each subgroup in SG2 (n=43)

5.2.3. What effect does timing of SO₂ addition have on MLFSG?

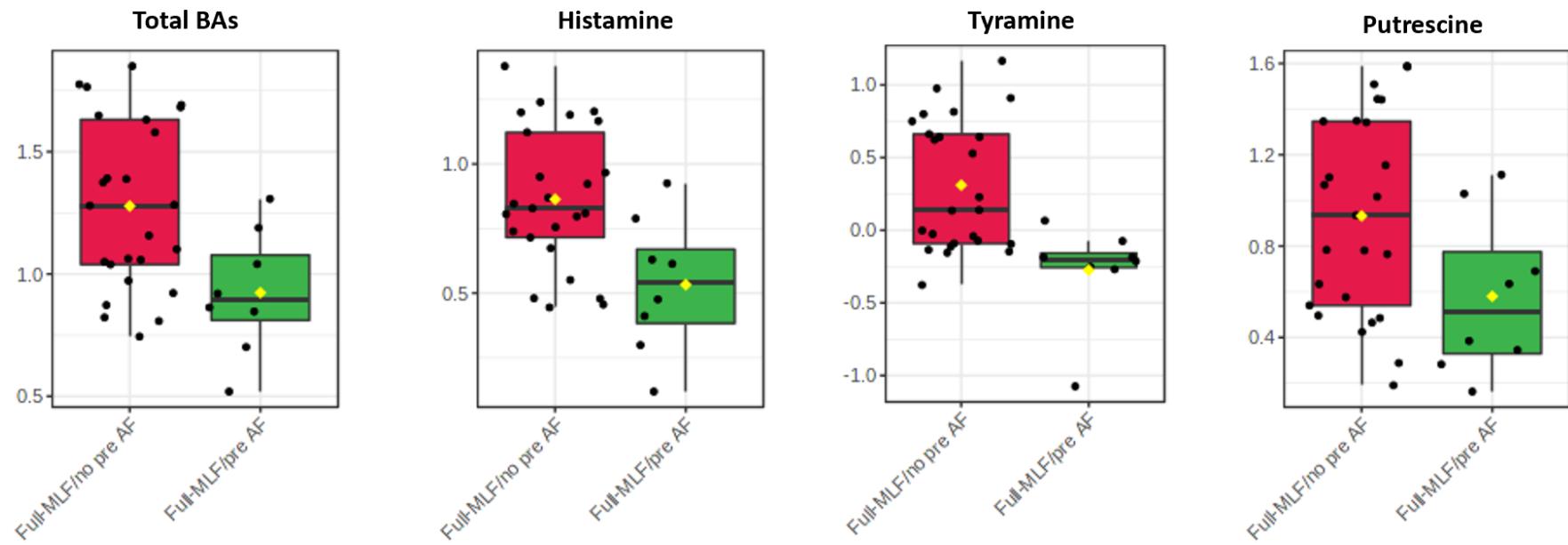
When MLFSG is further divided into subgroups depending on whether an S1 SO₂ addition was made or not, the results reveal that wines with an S1 addition had lower BA concentrations than S2–S4 and **zero-addition** wines (Figure 14).

Conducting t-tests for each of the subgroups, it was observed that in full-MLF samples, the fold-change values were >2-fold for all BA variables, and tyramine was >5-fold (Table 14). Conversely, TSO₂ was only 0.35-fold, which demonstrates that a pre-AF addition in wines destined for MLF will have BA concentrations 2–5-fold less than those that received no S1 addition.

	Fold change	log2 (FC)
Tyramine	5.31	2.4
Total SO ₂ addition	0.33	-1.84
Total SO ₂	0.35	-1.53
Total BAs	2.63	1.39
Putrescine	2.61	1.38
Histamine	2.21	1.14

Table 14 – T-test fold change values between pre-AF addition and no pre-AF addition of SO₂ in full-MLF samples (n=33)

14a)
Full-MLF
samples



14b)
No-MLF
samples

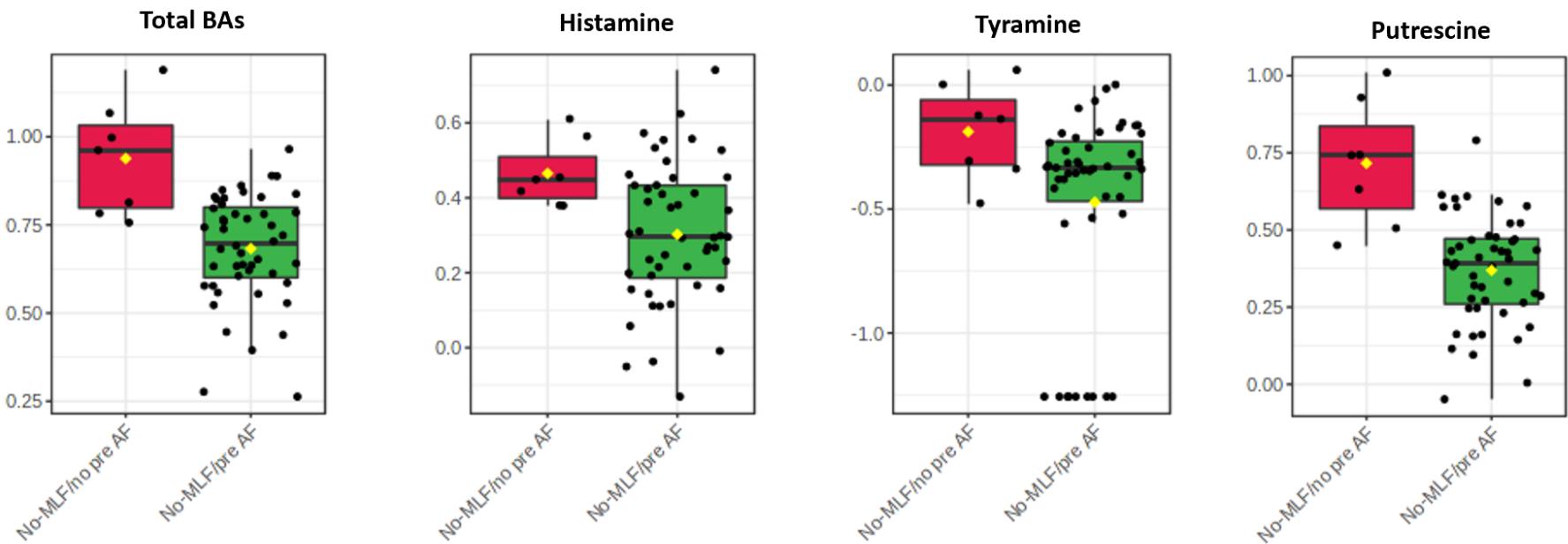


Figure 14 – Boxplots of BA concentrations for (a) full-MLF and (b) no-MLF samples, with pre-AF SO₂ addition (green) and without pre-AF SO₂ addition (red)
Y-axis represents normalised data 56

Thus, for wines destined for MLF, a pre-AF addition is crucial to minimise BAs because an S2 addition is not possible and S3 is too late to provide protection against decarboxylase-positive microbes. Furthermore, spontaneous MLF has a typically longer duration than inoculated MLF, which means an extended period of vulnerability to SO₂-sensitive decarboxylase-positive LAB. The corollary is that red wines, which ordinarily undergo MLF and have typically higher pH, should always have a pre-AF addition or risk high BA concentrations.

For non-MLF wines, an S1 **or** S2 addition will achieve similarly low BA concentrations. The literature establishes that post-AF onwards is where most growth occurs for LAB and therefore when most BAs accumulate. AF is nutrient-hungry and hugely efficient, preventing bacteria from growing as they cannot compete for nutrients. Consequently, the absence of SO₂ until immediately post-AF (S2) will not intensify BA accumulation in non-MLF wines because of the restricted opportunity for bacterial growth up until S2.

5.2.4. What is a sufficient S1 addition?

These findings are consistent with Wells and Osborne (2012), which demonstrated that bound SO₂ of 5–20 mg/L has a bacteriostatic effect on LAB such as *P. parvulus* and *L. hilgardii* (at pH values of 3.5 and 3.7). Transposing this into an equivalent SO₂ addition rate, an S1 addition of 30 mg/L to juice/must would typically result in ~15–20 mg/L TSO₂. However, machine-harvested Sauvignon Blanc typically receives a field SO₂ addition.¹⁸ Because of the oxidative nature of this, some SO₂ is oxidised to sulphate, so a higher addition rate (~60 mg/L) is required for field additions for a similar TSO₂ target. However, pH also needs to be considered when determining

¹⁸ This would generally be added in potassium metabisulphite (PMS) form. Some winemakers choose to make a field addition to other varieties also.

SO_2 addition rate as a higher pH reduces the effectiveness of SO_2 (Australian Wine Research Institute (AWRI) 2020b).

Although a smaller sample size, full-MLF samples receiving <30 mg/L SO_2 at S1 (must/juice) still resulted in relatively high BA concentrations. However, samples with an SO_2 addition ≥ 30 mg/L resulted in lower BA concentrations (Table 15), suggesting that <30 mg/L SO_2 at S1 is not enough because of the oxidation of SO_2 during this process.

Therefore, a pre-AF/S1 addition (≥ 30 mg/L at juice/must or ≥ 60 mg/L in field) is vitally important for minimising BA accumulation and is especially important for wines destined for MLF.

Full-MLF samples receiving S1 SO_2 addition	Total BAs	Histamine	Tyramine	Putrescine
<30 mg/L S1 addition ($n=2$)	14.3	4.36	1	8.93
≥ 30 mg/L S1 addition ($n=10$)	7.18	3.22	0.53	3.42

Values are mean concentrations.

Table 15 – Full-MLF samples receiving greater or less than 30 mg/L SO_2 addition at S1 ($n=12$)

5.2.5. S4 addition

The increase in BA concentrations between S4 and **zero-addition** is likely explained by SO_2 's inhibition of microbial activity post-bottling, which is likely to be ongoing in **zero-addition** wines that are unfiltered.

5.2.6. Outliers: green tea tannin

Two samples were removed from S2 as outliers in RQ2 because although they had no SO_2 addition at S1, they received an addition of green tea tannin, a relatively new

product marketed as an SO₂ alternative. Although these wines received SO₂ additions at later stages (S2 and S4), the low BA concentrations of these two wines (Table 16) suggests that green tea tannin has either a bactericidal or bacteriostatic effect which warrants further research.

Green tea samples	Total BAs	Histamine	Tyramine	Putrescine
24 ppm green tea at S1	2.27	0.81	0.31	1.15
23 ppm green tea at S1	2.99	1.28	LOQ	1.71

Table 16 – Analyses of BA concentrations in mg/L in samples using green tea tannin complex as an SO₂ alternative at S1 (n=2)

5.3. Relevance of style and other variables

Style and therefore SG1 subgroups must be considered. Table 17 shows that timing of first SO₂ addition and style overlap: **classic** and **alternative** styles are most likely to have early SO₂ additions (S1 or S2), which is consistent with the results for relative average BA concentrations across both SG1 and SG2. **Classic** wines, typically always having S1 addition, have the lowest average BA levels.

Number of samples in each SG1 subgroup receiving SO ₂ at each stage (S1 – S4)	Stage 1	Stage 2	Stage 3	Stage 4
Classic (n=30)	29	1	-	-
Alternative (n=48)	31	7	9	1*
Low-addition (n=12)	-	1**	1	10

* Technical information provided for this sample was inconsistent with the recorded TSO₂ concentration of the wine suggesting the producer had understated the SO₂ additions made.

** The addition at S2 for the low-addition wine was only 15 mg/L.

Table 17 – Distribution of when first SO₂ addition was first made to classic, alternative and low-addition samples in SG1 (n=90)

But most revealingly, **alternative** wines had a high distribution of other winemaking variables among the samples (percentages within the subgroup are indicated): full/partial-MLF (50%), skin contact (25%), lees ageing >6 months (81%), lees ageing >12 months (27%) and lees stirring (60%). This subgroup had the second lowest average BA concentrations, which were statistically similar to **classic** in tyramine concentrations and only 1.35 and 1.31 mg/L more on average in histamine and putrescine concentrations respectively. This confirms that these winemaking variables, thought of in the literature as significant factors for BA accumulation, are in fact not significant for the accumulation of BAs *unless* there is a zero or low TSO₂ environment.

Bentonite use was universal in **classic** wines and rare in **zero-addition** and **low-addition**, yet 20% of **alternative** wines also had no bentonite treatment. The data suggests that bentonite may have a small effect on resulting BA levels but is minor compared with SO₂ use.

5.4. Toxicity of samples

Applying the Esposito et al. (2019) threshold, 60% of **zero-addition** (*n*=6) and 17% of **low-addition** (*n*=2) wines exceeded the TBA concentration (31.21 mg/L). Two **zero-addition** wines exceeded every individual BA threshold (histamine – 9.98 mg/L, tyramine – 8 mg/L, putrescine – 13 mg/L). This is fewer because the tyramine threshold was high and consequently just three samples exceeded this.

However, these thresholds are not immutable, and this case study only serves as one precedent. It is likely the toxicity of samples within **zero-addition** and **low-addition** is greater than the Esposito thresholds suggest because in many cases, even though tyramine concentrations were <8 mg/L, histamine and putrescine

concentrations far exceeded the Esposito amounts. Applying both Menne et al. (2001) and Esposito's total dose-response limit of 4 mg histamine, a 300 mL dose of wine would cause toxicity in 40% of **zero-addition** ($n=4$)¹⁹ and 17% of **low-addition** ($n=2$) wines. None of the **alternative** or **classic** wines exceeded any of these concentrations and were on average, 23.69 and 26.62 mg/L below the TBA threshold, respectively.

¹⁹ One sample was 0.09 mg/L under the threshold and was therefore not included.

5.5. RQ3: Is there an argument for the creation of a low-BA category of wine?

There is genuine concern among researchers about BAs in F&B due to their toxic effects and quality implications. The results of this study demonstrate that the wine industry should also be very concerned about BAs in wine.

As section 2.2.2 states, ethanol and acetaldehyde's enzymatic inhibition enhances the risk of BA toxicity to wine consumers. This combined with individual sensitivities and the co-potentiation of BAs makes defining a specific BA toxicity threshold in wines challenging and has likely hindered regulation.

This section will explore whether a low-BA category of wine could be created to enable BA-sensitive consumers the opportunity to enjoy wines without risk of BA toxicity. Consideration of the regulatory landscape is necessary because claims regarding BAs on labels are regarded as general health claims, which are strictly regulated.

5.5.1. The regulatory landscape and challenges to the creation of a low-BA category

The regulatory landscape

The European Union (EU) continues to discuss introducing legal regulation for histamine in wine. Though no indication of a limit has been formally documented, a University of Bordeaux presentation mentioned a 10 mg/L limit had been proposed (Lucas 2014). The evidence demonstrates this may still be too high a threshold considering the co-potentiating factors and high correlation between the presence of histamine with other BAs.

On the other hand, imposing legal limits could burden the industry and ultimately the consumer with unreasonable additional compliance costs. RP HPLC methods are still the gold standard for BA analyses but the expense is considerable.²⁰ However, increased demand generally decreases cost, so a unified effort would assist in lowering costs.

Clearly an outright banning of BAs is unwarranted since not all consumers are affected by BAs in the same way. However, the creation of a low-BA category of wine, similar to the low-alcohol or vegan categories, would enable those consumers who have identified themselves as BA-sensitive to select and enjoy these wines without toxicity symptoms. Meanwhile the industry would avoid the burden and expense of blanket regulation of BAs.

Regulatory complexity exists with this category's creation because most countries' food standards codes prohibit health-related claims on alcoholic beverages²¹ unless the claim refers to the absence of substances that are expressly proscribed or limited by another standard.²² Claiming that a wine is low in BAs is deemed a health claim and because BA limits are not proscribed, it is not permitted.

This paradox of legislation versus public interest is exemplified by Vienna-based Schlumberger Aktiengesellschaft, who are intentionally following a low-histamine

²⁰ The only commercial laboratory capable of analysing for BAs quoted over USD\$260/wine, and only histamine and tyramine analyses were included in the service.

²¹ New Zealand/Australia, the UK and European Union food standards codes/legislation prohibits any health-related claims on alcoholic beverages. In the USA, the Bureau of Alcohol Tobacco and Firearms, which has jurisdiction over wine labelling, allows positive health claims on the proviso it presents balanced information regarding the negative effects of alcohol consumption.

²² For example, for the allergens regulated by legislation (EC) No: 607/2009 in the EU and Australia New Zealand Food Standards Code – Standard 1.2.3, producers would theoretically be allowed to state on the label that those allergens were absent in their wine.

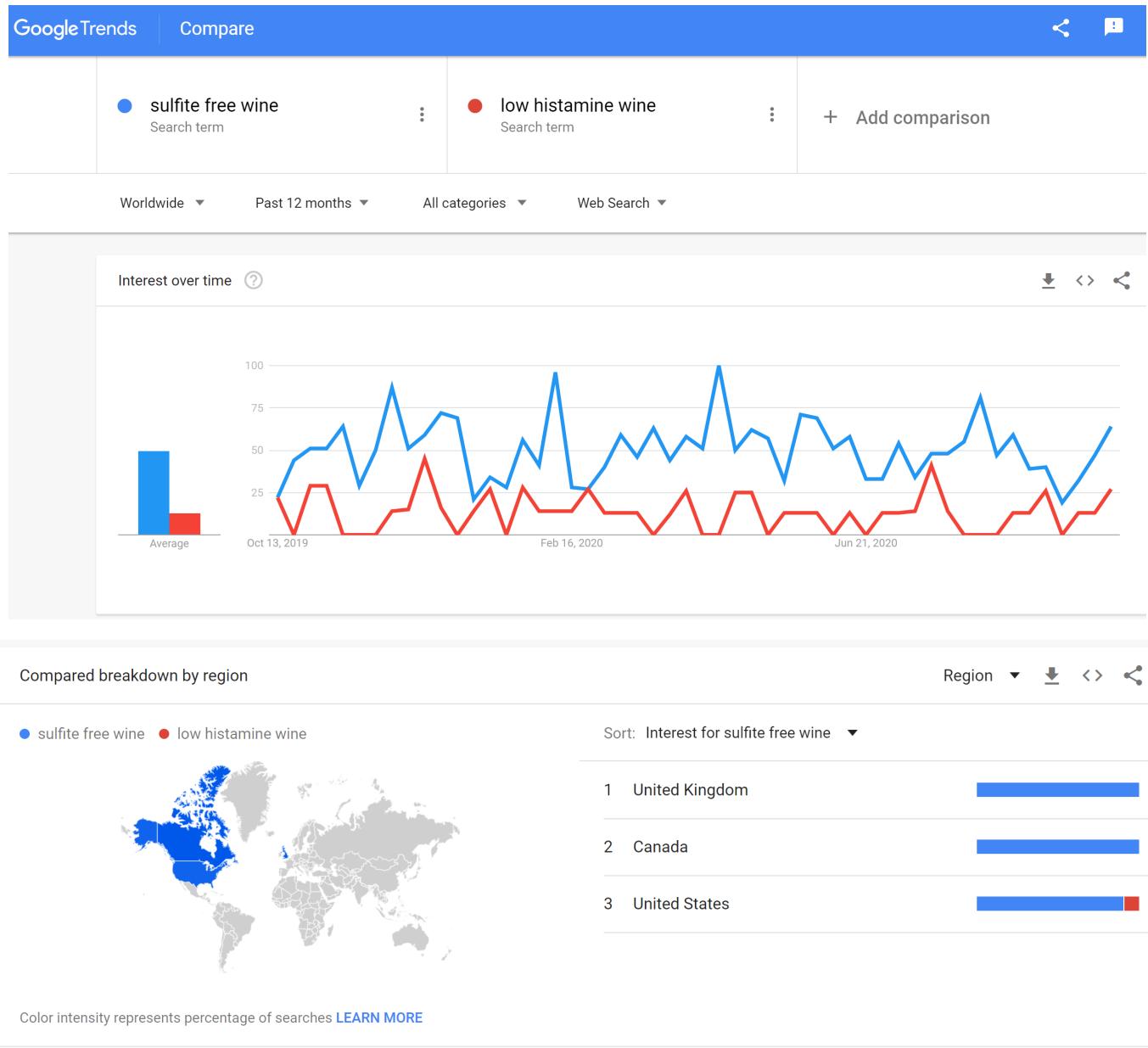
winemaking protocol yet cannot market their wines as being low in histamine
(Obritzhauser 2020 pers. comm.)

Current public interest in low/no SO₂

The results of this study have demonstrated that SO₂ is an important, if not the most important, factor in avoiding toxic BA levels in wine. Yet this conflicts with the current public interest in no-/low-SO₂ wines. Data on consumer attitudes toward SO₂ is limited, but a recent study found that 32% of UK wine consumers would be interested in natural wines made without any additives, including SO₂ (Kaczorowski 2019).

Examining Google Trends highlights the differing levels of public interest in low-/no-SO₂ versus low-/no-BA wines: ‘sulphite-free wine’ as a search term far outstrips that of ‘low-histamine wine’. Only the USA was shown to demonstrate any interest in the latter (albeit just 8% of searches) (Figure 15).

The need for public education on BAs and the opportunity to identify low-BA wines in the marketplace is unequivocal.



(Accessed 19/10/20)

Figure 15 - Google Trends data demonstrating indicative popularity of search terms 'sulfite free wine' verses 'low histamine wine' over a 12 month period and the breakdown of interest by country

5.5.2. Extent of the low-BA category opportunity

The epidemiological studies suggest that consumers who suffer from wine intolerance comprise as much as 10% of the general population (Wigand et al. 2012, Linneberg et al. 2008). As section 2 establishes, the likelihood that BAs are the primary agent in this response is high. These individuals may forgo wine in favour of other alcoholic beverages such as spirits or abstain from alcohol altogether, as may their partners. The social nature of wine and the conventional 750 mL bottle size means their abstention may also affect larger social groups.

Therefore, a low-BA category of wine has the potential to re-engage at least 10% of the general population.

5.5.3. Implementation options

Aside from enacting regulation on BAs, which, as discussed above, is likely to be difficult, there are two foreseeable ways to identify low-BA wines:

- 1) Concentrations of histamine, tyramine and polyamines could be stated on the wine label itself or via blockchain technology. However, the expense of analyses and labelling logistics, alongside consumer aversion to unfamiliar chemical terms on labels, suggests this could be impractical and off-putting.
- 2) An independent body could be created that would certify a low-BA winemaking regime (similar to the operation of organic/biodynamic bodies BioGro and Demeter). This would enable wineries to produce and label low-BA wines. Although this would take time and entail capital investment, a levy-funded not-for-profit organisation like this would allow for educational activities as well as further research on reducing BAs. Auditing would simply involve certified laboratory analysis.

From a policy perspective it is inconsistent to protect consumers through a label's sulphite declaration but not provide the same for BAs, especially when the risk to consumers' health is higher with the latter. Individuals who are sensitive to BAs (knowingly or unknowingly) have no way of knowing whether the wines they purchase will affect them adversely.

There is also a strong argument that wines with very high BA levels should carry a warning as these toxic levels pose a risk even to healthy individuals when moderate amounts of these wines are consumed. The results of this study show that wines made with no-/low-SO₂ are most likely to have the highest BA levels.

5.5.4. Conclusion

Whether regulation is implemented or not, the industry needs to act on the issue of BAs in wine, with two overarching priorities:

- i) Issue industry-wide guidelines to minimise BA accumulation in winemaking within the bounds of the style being made (addressed in section 6), in addition to the identification of low-BA wines to cater for individuals sensitive to BAs.
- ii) Increase public education to ensure both the industry and consumers are aware of BAs in F&B, and their likely role in wine intolerance. Mainstream media's cooperation is required to rectify the misinformation around wine intolerance, especially regarding the likelihood that sulphites are not the cause of most individuals' wine intolerance symptoms, unless that person is one of the 3–10% of acute asthmatics who suffer genuine sensitivity.

6. INDUSTRY RECOMMENDATIONS

6.1. Up-to-date guidelines

The absence of legal limits has retarded the industry's awareness of BAs. However, the literature and the results of this study establishes that industry-issued guidelines to address BA management in winemaking is required, especially with today's increasing number of low-/zero-SO₂ wines. This could come from any organisation with international standing.²³ The OIV published winemaking guidelines for BA management in 2011, yet its recommendations are relatively brief (RESOLUTION OIV-CST 369-2011). Most importantly, it emphasises grape health and advocates the use of MLF starter cultures to minimise BAs, but the usefulness of SO₂ is grossly understated. Revised guidelines are required.

Even Schlumberger, who employ a low-histamine winemaking protocol are unaware of the importance of early SO₂ addition (especially pre-AF) in minimising BA levels. Schlumberger state their use of commercial MLF starters for all their sparkling wines and adding SO₂ soon after completion is key to keeping histamine levels low (Obritzhauser 2020 pers. comm). However, it was only after specifically enquiring whether they added pre-AF SO₂ additions to their wines that it was revealed they did (at a rate of 50–60 mg/L)²⁴ but this was not communicated as a key part of their regime.

Figure 16 (pages 71–74) proposes up-to-date guidelines for BA management: a hierarchy of the most significant factors for BA accumulation and the

²³ Appropriate bodies might include respected research institutes such as the Australian Wine Research Institute (AWRI), or the International Organisation of Vine and Wine (OIV), by updating the 2011 code on BA management within the International Code of Oenological Practices (<http://www.oiv.int/en/technical-standards-and-documents/oenological-practices/international-code-of-oenological-practices>).

²⁴ An amount which the evidence and common knowledge suggests still allows for completion of MLF but is enough to inhibit decarboxylase-positive bacteria forming BAs.

practices/conditions that minimise or reduce their formation. It distils the literature to date and combines it with the findings of this study.

It demonstrates that SO₂ is the fundamental tool to control BA levels. Pre-AF additions (≥ 30 mg/L to must) will minimise BA accumulation, which is of greater importance if the wine is destined for MLF (because of the conditions during MLF that are favourable for general bacterial growth). A pre-AF addition resulting in >40 mg/L TSO₂ will likely maintain BAs at low- to moderate levels regardless of winemaking techniques used. Skin contact, lees ageing and lees stirring will all increase the free AAs in the wine. However, these are not an issue for BA accumulation unless coupled with favourable parameters such as low SO₂ and high pH, which will enable decarboxylase-positive microbes to produce BAs unchecked.

6.2. Communication about SO₂

The industry must address its communication regarding sulphites. Those producers who choose to follow a no- or low-SO₂ regime must ask themselves what health risks their products are (paradoxically) posing to their customers and must be careful their marketing does not contain misrepresentations regarding health claims.

6.3. Moral and legal obligations

There is a moral obligation and arguably a legal duty²⁵ to ensure wines produced contain minimal BA levels, until more specific thresholds are defined. If a zero-SO₂ style is still ultimately desired, producers should look to other viable alternatives to SO₂ such as the green tea tannin complex identified in RQ2, lysozyme or to a lesser

²⁵ There is both i) a statutory duty for producers to identify, control, manage and eliminate or minimise hazards and other risk factors in relation to the making of wine to ensure it is fit for its intended purpose (in individual countries' wine manufacturing legislation) and ii) a general duty of care under the tort of negligence. It is arguable the risk to consumers health from high BA concentrations in wine could potentially fall under both these causes of action.

extent dimethyl dicarbonate (DMDC).²⁶ If SO₂ additions are unconscionable for the Natural Wine movement perhaps zero-added SO₂ wines should carry a mandatory high-BA warning unless they can prove otherwise.

6.4. No-/low-SO₂ red wines

In this study the BA levels found in white wine with zero-SO₂ addition exceeded toxicological thresholds. They also exceeded the white wine BA concentrations found in other studies because those did not include zero-/low-SO₂ samples. As red wines typically contain higher BAs, then with a no-/low-SO₂ regime, spontaneous MLF and prolonged skin contact and lees ageing, what will be the results?

6.5. Conclusion

The issue of BAs needs to be brought into the open, and the industry needs to do its part to ensure that wines being sold are safe for consumers to drink. Starting the discussion and taking active steps to mitigate BA accumulation is the first step, followed by ensuring that those who suffer (knowingly or unknowingly) from BA sensitivity may reengage with wine. Many more research opportunities exist and it is imperative that the wine industry engages with researchers.

For winemakers it is reassuring that a relatively modest SO₂ regime, commencing with a pre-AF addition, will moderate resulting BA levels in wine, allowing freedom of winemaking style and creative expression in the cellar.

²⁶ All these alternatives may have legal restrictions in certain markets and have their own challenges in terms of application, suitability, legality and costs. There is consensus that DMDC is not as effective against wine bacteria as it is against wine yeasts. The maximum legal dose rate for DMDC is 200 mg/L (in NZ/Australia) which is not effective against all LAB (Australian Wine Research Institute (AWRI) 2020a).

Winemaking guidelines for BA management

AREA	SUB-AREA	PRACTICE / CONDITION / FACTOR	INFLUENCE ON BA PRODUCTION	DETAILS	SUPPORTING PAPER(S)
Microbial activity	Lactic acid bacteria (LAB)	Decarboxylase-positive LAB activity	++++	Decarboxylase-positive LAB, such as <i>P. parvulus</i> and <i>L. hilgardi</i> , are the most prolific BA producers.	Landete et al. 2007b
Wine parameters	Sulphur dioxide (SO ₂)	Zero or low SO ₂ additions	++++	Zero or low SO ₂ additions enable unrestrained microbial activity, including that of decarboxylase-positive and spoilage bacteria.	This study 2020
	pH	High pH	+++	Higher levels of BAs are produced at higher pH, especially pH >3.5, due to their favourability to bacteria.	Wibowo et al. 1985, Lonvaud-Funel and Joyeux 1994, Gardini et al. 2005, Landete et al. 2005a, Martín-Álvarez et al. 2006
Winemaking practices	Malolactic fermentation (MLF)	MLF, especially spontaneous MLF	+++	MLF facilitates the growth of LAB and encourages microbial growth. Spontaneous MLF has greater diversity of microbes, and increased risk of decarboxylase-positive activity.	Martin-Alvarez et al. 2006, Soufleros et al. 1998, Izquierdo Cañas et al. 2008, Granchi et al. 2005, Cilliers and Van Wyk 1985, Landete et al. 2005a, Marcobal et al. 2006, Alcaide-Hidalgo et al. 2007
	Maturation	Lees ageing	++	Overall concentration of BAs in wines matured with lees (>3 months) is higher with red and white wine, especially putrescine.	Martin-Alvarez et al. 2006, González-Marco and Ancín-Azpilicueta 2006b, Marques et al. 2008
	Lees stirring	Lees stirring	++	BAs increase with lees stirring – highest when stirred weekly, followed by monthly.	Alcaide-Hidalgo et al. 2007, Gonzalez-Marco and Ancín-Azpilicueta 2006a
	Maturation	Post-MLF maturation	++	Concentration of BAs, especially histamine, increase 4–18 months after MLF. Tyramine and putrescine increase immediately after MLF.	Herbert et al. 2005, Gerbaux and Monamy 2000, Jiménez-Moreno et al. 2003, Henriquez-Aedo et al. 2018, Marques et al. 2008
	Skin contact	Red wine maceration	++	Duration of skin contact increases BA formation due to AA liberation. Increase noted in skin contact >10 days.	Bauzá et al. 1995, Martín-Álvarez et al. 2006, Zee et al. 1983
		White wine maceration	++	Macerated white wine had highest BAs compared with free run or press cut wine.	Kovačević Ganić et al. 2009
Microbial activity	Yeast	<i>Brettanomyces bruxellensis</i> activity	++	Produced the highest total BAs of all yeasts studied – especially the BA 2-phenylethylamine.	Caruso et al. 2002, Granchi et al. 2005
	Yeast	Commercial and ambient yeast strain activity	+	Other strains (<i>S. cerevisiae</i>) can synthesise histamine, but amounts produced are minimal or negligible (study-dependent).	Caruso et al. 2002, Granchi et al. 2005, Torrea and Ancín 2002, Tristezza et al. 2013

AREA	SUB-AREA	PRACTICE / CONDITION / FACTOR	INFLUENCE ON BA PRODUCTION	DETAILS	SUPPORTING PAPER(S)
Storage	Storage	Wine storage in bulk	+	Decarboxylase enzymes are active even after bacteria population is non-viable thus BAs can still accumulate. After initial increase of BAs during storage a general decrease or stabilisation in concentration generally occurs.	Coton et al. 1998, Gerbaux and Monamy 2000, Landete 2005a, Marcobal et al. 2006
	Storage	Wine storage in bottle	+	BAs can increase further in bottle. Histamine increased during first 6 months in bottle before decreasing. Other BAs were stable.	Landete et al. 2005a, Gonzalez-Marco and Ancín-Azpilicueta 2006b
Winemaking practices	Additions	Yeast mannanprotein use	+	BAs increased after addition of yeast mannanprotein.	Garcia-Marino et al. 2010
	Pressing	Press wine	+	Press wine had higher BAs than free run wine (27% more), however press wine was added to free run after MLF before analysis which may have contributed to higher levels.	Garcia-Marino et al. 2010
	Malolactic fermentation (MLF)	Inoculated MLF	+	No new BAs produced after inoculated MLF, a significant but slight increase in histamine was observed.	Garcia-Marino et al. 2010
Wine parameters	Ethanol	Low ethanol ≤ 10%	+	HDC activity is enhanced.	Lonvaud-Funel and Joyeux 1994
	Sugars	Low glucose / fructose environment	+	Lack of sugars are associated with higher BA production.	Landete et al. 2006
Viticulture	Fungi	<i>Botrytis cinerea</i> activity	+	Can lead to an increase of BA content in grape berries.	Hajos et al. 2000, Sass-Kiss et al. 2000
Winemaking practices	Alcoholic fermentation (AF)	Alcoholic fermentation	+/-	Decrease, no change or slight increase in BAs during AF. AF is not a significant factor for BA production (unless spoilage yeasts present).	Herbert et al. 2005, Marcobal et al. 2006, Granchi et al. 2005, Vidal-Carou et al. 1990b
	Storage	Wine storage temperature	+/-	Temperature has a minor impact on BA concentration. Histamine increased slightly when wines were stored at 20°C (rather than extreme temperatures of 4°C or 35°C).	Smit et al. 2008, Gonzalez Marco and Ancín-Azpilicueta 2006b, Marcobal et al. 2006
Wine parameters	Malic acid	Malic acid presence	+/-	HDC gene expression is reduced, but activates arginine catabolism, increasing putrescine.	Landete et al. 2006
	Lactic acid	Lactic acid presence	+/-	Inhibits HDC but not ODC activity.	Rollan et al. 1995, Lonvaud-Funel 2001, Mangani et al. 2005
Winemaking practices	Additions	Pectolytic enzyme use	± (Neutral)	Addition of pectolytic enzymes to grapes did not promote BAs.	Martin-Alvarez et al. 2006, Smit et al. 2013
	Additions	Nitrogen-containing supplement use	± (Neutral)	Addition of nitrogen-supplements (fermentation aids) did not promote BAs.	Marques et al. 2008

AREA	SUB-AREA	PRACTICE / CONDITION / FACTOR	INFLUENCE ON BA PRODUCTION	DETAILS	SUPPORTING PAPER(S)
Winemaking practices	Additions	Nitrogen-containing supplement use	± (Neutral)	Addition of nitrogen-supplements (fermentation aids) did not promote BAs.	Marques et al. 2008
	Maturation	Turbidity of wine	± (Neutral)	Degree of turbidity (gross/fine lees) did not influence BA content during ageing.	Moreno and Azpilicueta 2004
	Maturation vessel	Barrel use	± (Neutral)	Barrels and barrel type do not influence the content of BAs.	Moreno and Azpilicueta 2004
	Treatments	Pasteurization	± (Neutral)	Decarboxylase enzymes and BAs are heat stable and are not reduced during processing treatments such as pasteurization.	ten Brink et al. 1990
Wine parameters	Acetic acid bacteria	Acetic acid bacteria activity	± (Neutral)	No positive results obtained from 40 strains of acetic acid bacteria isolated from must/wine.	Landete et al. 2007
Viticulture	Viticulture treatments	Fungicide application in vineyard	-	Fungicides applied to vines every three weeks reduced BA incidence in must and wine.	Marques et al. 2008
Wine parameters	Citric acid	Citric acid presence	-	HDC and TDC activity inhibited to a small extent at levels normally present in wines post-MLF.	Rollan et al. 1995, Moreno-Arribas and Lonvaud-Funel 1999
Winemaking practices	Ethanol	High ethanol >12% abv	-	Reduces HDC activity.	Rollan et al. 1995
	Phenolics	Presence of phenolics	-	Phenolic compounds (except gallic acid and quercetin) reduced putrescine formation.	Alberto et al. 2007, Garcia-Ruiz et al. 2007
Winemaking practices	Additions	Dimethyl decarbonate (DMDC)	-	DMDC has anti-microbial properties but it is not as effective against wine bacteria as it is against wine yeast, requiring higher dosage rates to kill the former. It will kill some LAB at ≥200 mg/L and therefore curb BA formation.	Australian Wine Research Institute (AWRI) 2020a
	Additions	Bentonite use	--	Bentonite can reduce BA levels by absorption, with studies showing it effective at both must stage and pre-bottling. However bentonite's removal of colouring matter makes its use in red wine less desirable.	Schneyder 1983, Fehrenbach 2020
Winemaking practices	MLF	LAB starter culture inoculation	--	Inoculation with <i>O. oeni</i> starter cultures that are unable to produce BAs can curb BA formation.	Martin-Alvarez et al. 2006
	MLF	Co-inoculation of MLF	--	Co-inoculation of <i>O. oeni</i> starters together with alcoholic fermentation can curb BA formation.	Van der Merwe 2007
Wine parameters	pH	Low pH level	---	Lower pH level <3.3 makes environment more hostile for bacteria and MLF, thus BA-accumulation less likely.	Gardini et al. 2005

AREA	SUB-AREA	PRACTICE / CONDITION / FACTOR	INFLUENCE ON BA PRODUCTION	DETAILS	SUPPORTING PAPER(S)
Winemaking practices	Additions	Lysozyme use	----	Causes lysis of cell walls of gram-positive bacteria. Can delay or inhibit growth of most LAB.	Smit et al. 2008
	Additions	SO ₂ use	----	Total SO ₂ prevents the formation of BAs by inhibiting decarboxylase-positive microorganisms. Modest amounts of TSO ₂ potently inhibit BA producing bacteria.	Marcabal et al. 2006, Vidal-Carou et al.1990a, Wells and Osborne 2012, this study 2020
	Additions	Pre-AF SO ₂ addition	----	A pre-AF addition of ≥30 mg/L to juice/must or ≥60 mg/L in the field will minimise BA accumulation by inhibiting decarboxylase-positive SO ₂ -sensitive microbes, especially for wines destined for MLF.	This study 2020

Figure 16 key:

+, ++, +++, ++++	increasing influence on BA production (in order of influence)
+/-	increasing or decreasing influence (BA dependent or conflicting results in studies)
± (Neutral)	no influence on BA production
-, --, ---, ----	decreasing influence on BA production (in order of influence)

Figure 8 abbreviations

AA	Amino acid
AF	Alcoholic fermentation
BA	Biogenic amines
DMDC	Dimethyl dicarbonate
HDC	Histidine decarboxylase
LAB	Lactic acid bacteria
TDC	Tyrosine decarboxylase
MLF	Malolactic fermentation
ODC	Ornithine decarboxylase
TSO ₂	Total sulphur dioxide

Figure 16 - Winemaking guidelines for BA management: hierarchy of conditions/practices that influence BA production in wine

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Appendix 1: Abbreviations

AA	Amino acid
AF	Alcoholic fermentation
ANOVA	Analysis of variance
AWRI	Australian Wine Research Institute
BAs	Biogenic amines
DBPC	Double-blind placebo-controlled
DMDC	Dimethyl dicarbonate
F&B	Food and beverage
FDR	Adjusted p-values
FSO ₂	Free sulphur dioxide
HCl	Hydrochloric acid
HDC	Histidine decarboxylase
HPLC	High-pressure liquid chromatography
IgE	Immunoglobulin E
LAB	Lactic acid bacteria
LOQ	Limit of quantification
LSD	Least significant difference
MLF	Malolactic fermentation
MLFSG	Malolactic fermentation sample group
NAC	N-acetyl-L-cysteine
ND	Not detectable
ODC	Ornithine decarboxylase
OIV	International Organisation of Vine and Wine
OPA	o-phthalodialdehyde
PCA	Principal component analysis
RP	Reverse phase
RQ1	Research question 1
RQ2	Research question 2
RQ3	Research question 3
SG1	Sample group 1
SG2	Sample group 2
SO ₂	Sulphur dioxide
TBA	Total biogenic amines
TDC	Tyrosine decarboxylase
TSO ₂	Total sulphur dioxide

Appendix 2: Medications inhibiting histamine detoxification

List of medications that inhibit enzymatic detoxification of histamine (derived from Swiss Interest Group Histamine Intolerance (SIGHI), available online https://www.histaminintoleranz.ch/en/therapy_medicaments.html updated 10/05/2020 (Accessed 05/10/2020)

Active substance	Examples of products ®	Categories	Histamine effects
Acemetacin		Antirheumatic	DAO inhibitor
Acetylcysteine	Fluimucil, Helvetussin, Muco-Mepha, NeoCitran, Solmucol	Mucolytic, antidote	DAO inhibitor
Acetylsalicylic acid, ASS	Aspirin	Analgesic	Histamine liberator
Acriflavin		Antiseptic	DAO inhibitor
Alcuronium		Muscle relaxant	DAO inhibitor
Alprenolol		Beta blocker	DAO inhibitor
Ambroxol	Ambrovene, Ambroxol, Broxol, Mucosolvan, Mucospas	Expectorant	DAO inhibitor
Amiloride		Diuretic	Uncertain
Aminocycline			DAO inhibitor
Aminophyllin	Euphyllin, Mundiphyllin, Myocardon	Antiasthmatic	DAO inhibitor
Amiphenazole			DAO inhibitor
Amitriptyline	Saroten, Tryptizol, Limbritol	Tricyclic antidepressant	DAO inhibitor
Amodiaquins		Antimalarials	HNMT-Blocker
Amphetamine			Histamine liberator
Amphotericin B		Antibiotic	Histamine liberator
Atracurium		Muscle relaxant	Histamine liberator
Atropine			Histamine liberator
Barbiturates		Hypnotics, sedatives, anaesthetics	Histamine liberator

Active substance	Examples of products ®	Categories	Histamine effects
Bile acids, bile salts			Histamine liberator
Bupropion		NDRI	Histamine liberator
Carbamazepine		Anticonvulsant	Histamine liberator
Carbocromene			DAO inhibitor
Cefotiam		Antibiotic	DAO inhibitor
Cefuroxime		Antibiotic	Histamine liberator
Chloroquine	Chlorochin, Nivaquine, Resochin	Antimalarials, antirheumatic	DAO inhibitor, HNMT-Blocker
Chlortetracyclins		Antibiotic	Histamine liberator
Cimetidine		H2 antihistamine	DAO inhibitor
Ciprofloxacin		Antibiotic	Histamine liberator
Clavulanic acid	Augmentin	Antibiotic	DAO inhibitor
Codeine		Opiate, analgesic, cough medicine	Histamine liberator
Colistin mesilate			DAO inhibitor
Curare		Arrow poison alkaloids, anaesthetic	
Cyclophosphamide		Cytostatic	Uncertain
D-Cycloserine	Seromycin	Antibiotic	DAO inhibitor (Vitamin B6-Antagonist)
Decamethonium			Histamine liberator
Dextranes	Sephadex	Blood plasma substitute, antithrombotic	Histamine liberator
Diazepam	Valium	Tranquilizer	DAO inhibitor

Active substance	Examples of products ®	Categories	Histamine effects
Diclofenac	Voltaren	Antirheumatic	Histamine liberator
Dihydralazine	Nepresol	Antihypertensive, vasodilator	DAO inhibitor
Diphenhydramine	Nardyl, Benocten	Sedative, antihistamine	HNMT-Blocker
Dipyrrone: see Metamizol			
Dobutamine		Antihypotonic	Uncertain
Fenpiverinium			DAO inhibitor
Flurbiprofen			Histamine liberator
Framycetin		Antibiotic	DAO inhibitor
Furosemide	Lasix	Diuretic	DAO inhibitor
Gadolinium chelates		X-ray contrast media	Histamine liberator
Gallamines			Histamine liberator
Gelatine		Plasma substitute	Histamine liberator
Glyceroltrinitrate, glycercyltrinitrate, nitroglycerin, Propantriol-trinitrat		Vasodilator	Histamine liberator
Haloperidol	Haldol	Neuroleptic	DAO inhibitor
Heroin			Histamine liberator
Hydralazine			Histamine liberator
Hydroxyethyl starch		Plasma substitute	Histamine liberator
Indomethacin			Histamine liberator

Active substance	Examples of products ®	Categories	Histamine effects
Isoniazide	Rimifon, Rifater	Tuberculostatic	DAO inhibitor (Vitamin B6-Antagonist)
Ketoprofen			Histamine liberator
Latex gloves			Histamine liberator
Levofloxacin		Antibiotic	Histamine liberator
Meclofenamic acid			Histamine liberator
Mefenamic acid			
Meperidine: see pethidine		Opioid	
Metamizole, dipyrone	Novalgin, Minalgin	Analgesic, antipyretic	DAO inhibitor
Methohexital		Injection narcotic	Histamine liberator
Metoclopramide	Migpriv, Paspertin, Primperan	Antiemetic, gastroenterologic, dopamine antagonist	DAO inhibitor
Metoprine			HNMT-Blocker
Mivacurium		Muscle relaxant	Histamine liberator
Morphine		Analgesic, opioid	Histamine liberator
Naproxen			Histamine liberator
Nefopam		Analgesic	Histamine liberator
Neomycin		Antibiotic	DAO inhibitor
Nitroglycerin, glycerol trinitrate, glycercyl trinitrate, propane triol trinitrate		Vasodilator	Histamine liberator

Active substance	Examples of products ®	Categories	Histamine effects
Nonsteroidal anti-inflammatory drugs (NSAID)		Analgesic	Histamine liberator
Noscapine		Analgesic	Histamine liberator
Novamine sulfone	(=Metamizol) Novalgin, Minalgin	Analgesic, antipyretic	DAO inhibitor
NSAP, NSAR, NSAID	Verträglichkeit individuell!	NSAR, NSAID	(Histamine liberator)
Opiates, Opioids	(Heroin, Morphium)	Analgesic	Histamine liberator
Orciprenaline			DAO inhibitor
Pancuronium		Muscle relaxant	DAO inhibitor
Papaverine			Histamine liberator
Pentamidine		Antibiotic	DAO inhibitor
Pethidine, Meperidine		Analgesic, opioid	Histamine liberator
Phenobarbital		Injection narcotic	Histamine liberator
Pilocarpine			Histamine liberator
Pirenzepine			DAO inhibitor
Polymyxin B		Antibiotic	Histamine liberator
Polyvinylpyrrolidone (E1201)	Povidone, PVP, Periston	Blood plasma substitute, antithrombotic, excipient in tablets and capsules	Histamine liberator
Prilocaine		Local anaesthetic	DAO inhibitor
Procaine		Local anaesthetic	Histamine liberator
Promethazine	Atosil, Closin, Proneurin, Prothazin	Sedative, antihistamine, antipsychotic	DAO inhibitor
Propafenone	Rytmonorm	Antiarrhythmic	DAO inhibitor

Active substance	Examples of products ®	Categories	Histamine effects
Propanidide		Anaesthetic	DAO inhibitor
Protamine		Heparin antagonization	Histamine liberator
Pyrazolones		Analgesic	Histamine liberator
Quinidine		Cardiac	DAO inhibitor
Quinine			Histamine liberator
Reserpine			Histamine liberator
Rifampicin		Antibiotic	Histamine liberator
Rifaximin		Antibiotic	Histamine liberator
Scopolamine			Histamine liberator
SSRIs, selective serotonin reuptake inhibitors		Antidepressant	Unverträglich
Stilbamidine			Histamine liberator
Suxamethonium		Muscle relaxant	Histamine liberator
Tacrin		Acetylcholinesterase inhibitor, Alzheimer's drug	HNMT-Blocker
Teicoplanin		Antibiotic	Histamine liberator
Tetracaine		Local anaesthetic	Histamine liberator
Tetroxoprim			DAO inhibitor
Thiamine (vitamin B1)	(In parenteral administration. Compatible with food.)		Histaminliberator, DAO inhibitor
Thiopental		Sedative	Histaminliberator / DAO inhibitor
Tolazoline			Histamine liberator
Topiramate		Migraine, anti-epileptic	Histamine liberator

Active substance	Examples of products ®	Categories	Histamine effects
tricyclic antidepressants			Unverträglich
Tubocurarins, D-Tubocurarin		Muscle relaxant	Histamine liberator
Tyramine			Histamine liberator
Vancomycine		Antibiotic	Histamine liberator
Verapamil	Flamon, Isoptin, Tarka	Coronaryvasodilatant, antihypertensive, antiarrhythmic, calcium antagonist	DAO inhibitor
X-ray contrast media	All of them!	X-ray contrast media	Histamine liberator
β-Adrenoceptor blockers			Histamine liberator

List of medications that inhibit enzymatic detoxification of histamine (derived from Swiss Interest Group Histamine Intolerance (SIGHI), available online

https://www.histaminintoleranz.ch/en/therapy_medicaments.html updated 10/05/2020 (Accessed 05/10/2020)

Appendix 3: BA toxicity thresholds in wine

HISTAMINE TOXICITY THRESHOLDS IN WINE			
Reference	Histamine concentration (mg/L) eliciting symptoms and volume of wine consumed	Total dose equivalent of histamine eliciting symptoms (mg)	Critique / comments
Clinical Studies: Scientifically sound designs – Double-Blind Placebo Controlled Design (DBPC)			
Menne et al. 2001	20 mg/L in susceptible patients after 200 mL of sparkling wine	4 mg	<p>Study: 40 patients with suspected histamine intolerance were challenged with an oral histamine provocation test in sparkling wine. Dose comprised 20 mg/L histamine in a 200 mL wine sample. 12/40 patients demonstrated clear clinical symptoms after the histamine-rich wine, with no reaction to the placebo. Only one patient reacted positively to the placebo.</p> <p>Design: Study was DBPC designed but it did not consider co-potentiating factors such as ethanol (due to small amount of wine consumed) or other BA co-potentiators.</p>
Clinical Studies: Methodology not specified / available (uncertain whether scientifically sound)			
Battaglia and Frolich 1978	5 mg/L after the consumption of 500 mL of wine	2.5 mg	<p>Claim: <i>"if the concentration is higher than ca.5 mg/l the wine is almost certain to cause headache when consumed in quantities higher than 0.5 litres."</i></p> <p>Design: Did not state any evidence or reference for the threshold given.</p>
Schuller et al. 1967 cited in Schneyder 1973	2 mg/L in wine <i>Volume of wine not specified</i>	<i>Not specified</i>	<p>Claim: <i>"The maximum amount of histamine tolerable in wine for a male adult is 2 mg / L."</i></p> <p>Design: Study and methodology details were not provided.</p>
Lehtonen et al. 1992	5 mg/L in wine <i>Volume of wine not specified</i>	<i>Not specified</i>	<p>Claim: <i>"[At 5 mg/L histamine in wine]... it is almost certain to give rise to physiological effects."</i></p> <p>Design: Study and methodology details were not provided. It did however acknowledge co-potentiating factors must be considered in addition to this.</p>
Case study			
Esposito et al. 2019	9.97 mg/L histamine 8.23 mg/L tyramine 13.01 mg/L putrescine after consumption of 400 mL of wine	4 mg histamine 3.3 mg tyramine 5.2 mg putrescine	A case study (not a clinical trial) of strongly suspected BA poisoning in six young adults who consumed ~3 glasses (400 mL) of sparkling red wine on tap. The symptoms required hospitalisation of subjects after the consumption of wine.

Positive correlation clinical studies: design not scientifically sound (not DBPC or other design failure)			
Wantke et al. 1994	0.4 mg/L in 125 mL red wine (sensitive individuals)	0.05 mg	<p>Study and results: Included 18 patients with history of wine intolerance and 10 healthy controls. Blood samples were drawn to measure plasma histamine. 22/28 showed higher plasma histamine levels 30 mins after wine challenge compared to asymptomatic controls. Basal histamine levels were higher than controls. 5/28 patients suffered headache with 0.05 mg histamine in 125 mL red wine.</p> <p>Design: Study was not DBPC; however, it is questionable whether it needed to be because testing of blood plasma is an objective clinical test. Did not entertain any co-potentiating factors (presence of ethanol or acetaldehyde) due to small volume of wine administered.</p>
Wantke et al. 1996	0.2 mg/L (histamine-poor wine) 3.7 mg/L (histamine-rich wine) in 125 mL red wine (sensitive individual)	0.025 mg (histamine-poor) 0.46 mg (histamine-rich)	<p>Study and results: A case report of a 38-year-old woman with seasonal rhinoconjunctivitis reporting repeated attacks of wheezing after drinking various alcoholic beverages. Two consecutive histamine provocations were conducted using two identical samples of red wine. DBPC design assessing lung function, plasma histamine, skin temperature, pulse rate and symptoms was conducted. Four consecutive tests were performed on 3 controls in DBRPC design. Drinking wine with 3.7 mg/L histamine caused coughing and wheezing with a decrease in lung function. Plasma histamine increased with both histamine-rich and histamine-poor wine with peak increase after histamine-rich wine. Controls did not react. Concluded that histamine may induce bronchoconstriction in patients with histamine intolerance.</p> <p>Design: Study was DBPC but was criticised by Jansen et al. (2001) for not following an <i>n=1</i> design. Did not include co-potentiating factors (ethanol) due to small amount of wine administered, nor BA co-potentiators.</p>
Negative correlation clinical studies: design not scientifically sound (not DBPC or other design failure)			
Lüthy and Schlatter 1983	mg/L dose BAs not specified. 200 mL of wine – red and white wines used as samples	0.12–4.2 mg	<p>Study and results: 20 healthy subjects consumed 200 ml of wine with varying amounts of BAs (histamine ND–21ppm, tyramine 1–23ppm, phenylethylamine ND–6ppm, putrescine 2–55ppm). Results only found phenylethylamine had an effect with positive headache correlation (5 mg dose equivalent). Results did not find a statistically significant effect with other BAs.</p> <p>Design: The number of positive responses was not indicated. A high response to placebos in the study, which makes results inconclusive in accordance with Chi et al. (2015). Study did not entertain any co-potentiating factors (presence of ethanol or acetaldehyde) due to small amount of wine administered.</p>

Dahl et al. 1986	8.5–9.5 mg/L of amines (unspecified) with incremental small amounts of wine consumed at 15-minute intervals to a total of 385 mL red wine over an hour	2.76–3.09 mg of amine.	<p>Study and Results: Included 18 patients (10 men and 8 women) with a recent history of red-wine provoked asthma. 8 patients had IgE-mediated response to red wine, 10 were non-allergic. Results were based exclusively on lung-function. The wine was a 1981 Châteauneuf-du-Pape with adjusted SO₂ and amines to suit the experiment:</p> <ul style="list-style-type: none"> I) Original wine had a free SO₂ (FSO₂) 6–7 mg/L, a total SO₂ (TSO₂) 50–55 mg/L and a ‘high amine content’ 8.5–9.5 mg/L. II) High SO₂ wine had a FSO₂ 180–190 mg/L and TSO₂ 265–275 mg/L. III) Low amine wine (mg/L not specified). <p>9/18 responded to one or more of the challenges; 4/18 responded to two or all of challenges. 5/18 reacted only to the high SO₂ wine. No statistical significance between BAs and positive asthmatic response. Statistical significance to high SO₂ and positive asthmatic response. 8/18 had no reaction.</p> <p>Design: No placebo was used even though double-blind, randomized design was used. A confused study because of patient selection (all asthmatics), looking exclusively at lung function (through FEV values). The high SO₂ wine was extremely high in concentrations (illegal by today’s standards), while design for amines was inappropriate as the amine was not specified and the amine content was not specified in the low amine wine. Sensitive individuals included only. Did not include consideration of BA co-potentiators.</p>
Kanny et al. 2001	13.8 mg/L histamine (histamine-rich wine) in 190 mL of red wine 0.4 mg/L histamine (histamine-poor wine) in 190 mL of red wine	2.6 mg for histamine-rich wine	<p>Study and Results: 16 patients, all suffering self-diagnosed wine intolerance. Subjects reacted to both wines: 14/16 to the histamine-poor wine and 15/16 to the histamine-rich wine.</p> <p>Design: A high response to the placebo (87%) suggested the Chi et al. (2015) principle needed to apply. Study did not include co-potentiating factors (ethanol) due to the small amount of wine administered, nor BA co-potentiators. Included sensitive individuals only. The histamine-poor wine content (0.4 mg/L) – the placebo - was enough to provoke headache and other symptoms in subjects of other studies so the placebo was not necessarily appropriate and may have resulted in the high response rate to the ‘placebo’ (Panconesi 2008).</p>

TYRAMINE TOXICITY THRESHOLDS IN WINE			
Littlewood et al. 1988	2 mg/L tyramine in 300 mL of red wine	<1 mg tyramine	<p>Study and Results: 24 migraine patients - 19 selected because of belief their headaches could be provoked by red wine, not by vodka/gin. Challenged with either 300 mL red wine or 300 mL vodka lemon mixture (placebo). Both drinks were chilled “obscuring flavour” and served in brown glass bottles with dark straws to conceal colour. 11/19 red wine sensitive patients received red wine; 8 patients received placebo. 5 migraine patients who were not sensitive to red wine and 8 controls received placebo. The clear effect of red wine could be seen: 9/11 red wine sensitive patients developed headache after red wine vs. 0/8 red wine sensitive migraine patients after vodka. No other migraine patients or controls developed headache. Littlewood stated positive results were therefore not caused by tyramine (because the concentration was too low), and it was not alcohol itself because of positive responses only to the red wine (not to the placebo).</p> <p>Design: The blinding of the study was questionable because of the taste difference (even if chilled) (Jansen et al. 2001). No co-potentiating factors were considered (BAs or ethanol). Therefore it does not definitively prove tyramine is not a factor in red-wine induced migraine.</p>

Appendix 4: Technical data template

BIOGENIC AMINE STUDY - TECHNICAL INFORMATION TEMPLATE FOR WINE SAMPLES	
Wine name	
Vintage	
Variety / blend (if not 100% Sauvignon Blanc)	
pH	
Was pH adjusted? If yes, when?	
Nitrogen-supplements added? If yes, type and amount added	
Skin-contact? If yes, how long?	
Inoculated or wild primary fermentation?	
Fermentation vessel?	
MLF? (Y/N) if yes, full or partial?	
Inoculated or spontaneous MLF?	
Lees contact? (Y/N) If yes, length of time on lees	
Lees stirring? (Y/N) If yes, how often?	
Bentonite added? (Y/N)	
Proteinaceous fining? (Y/N) If yes, fining agent + amount used	
Filtered? If yes, type and when	
SO₂ regime (<i>please indicate with an X if zero-addition, or with the addition amounts in mg/L as SO₂ equivalent next to the appropriate stage</i>)	
Zero-addition SO ₂ (Y/N)	
Mg/L SO ₂ before fermentation (pre-AF)	
Mg/L SO ₂ post-fermentation (post-AF)	
Mg/L SO ₂ post-MLF (if applicable) or post-assemblage	
Mg/L SO ₂ pre-bottling	
Total SO ₂ (at bottling)	
Free SO ₂ (at bottling)	
Date bottled	

Appendix 5: HPLC protocol

HPLC Protocol

The following details the reverse phase (RP) high pressure liquid chromatography (HPLC) protocol for analysis of biogenic amines in wine samples, adapted from Kelly et al. (2010).

1. Reagents and solutions

All chemicals and reagents were of analytical, HPLC grade or equivalent. Methanol, acetonitrile, acetic acid, sodium acetate, potassium chloride, boric acid, hydrochloric acid 0.1 M (HCl) and sodium hydroxide 1 M were obtained from ThermoFisher Scientific (Lithuania), FLUKA (USA), and Sigma-Aldrich (Germany). The three BAs, *o*-phthaldialdehyde (OPA) and N-acetyl-L-cysteine (NAC) were purchased from Sigma-Aldrich (Germany). Ultrapure water was used to prepare solutions and for washing all consumable materials.

Individual stock solutions of each analyte (4 g/L) were made with 0.1 M HCl. A working mixed-stock solution containing 40 mg/L of the analytes was prepared in ultrapure water on a weekly basis. Calibration standards were prepared by serial dilution of the working mixed-stock solution in ultrapure water made to 10 mL each and filtered (0.45 µm syringe filter) into HPLC vials (Table A).

A mixed control standard was prepared by pipetting 1 mL of the working mixed-stock solution (40 mg/L) into a 10 mL volumetric flask made to 10 mL with ultrapure water and filtered (0.45 µm syringe filter) into clear HPLC vials.

Calibration	Volume (mg/L)	Working mixed-stock solution volume (mL)
1	0.25	0.0625
2	0.5	0.125
3	1	0.25
4	2	0.5
5	5	1.25
6	10	2.5

Table A – Calibration standards for HPLC protocol

2. Determination of BAs

i) Derivatisation reagent and sample preparation

The derivatisation reagent consisted of 2.5 mL NAC solution and 0.5 mL OPA solution (Table B). The derivatisation reagent was prepared daily and stabilised at room temperature for 90 minutes before use. Both OPA and NAC solutions were kept refrigerated, renewed after 10 days of use.

Solution	Contents
NAC Solution	400 mg NAC dissolved in 50 mL of a 0.2 M borate buffer adjusted to pH 9.5 with sodium hydroxide
OPA Solution	50 mg of OPA dissolved in 10 mL methanol

Table B – NAC and OPA solutions for HPLC protocol

Unfiltered wines were centrifuged prior to analysis. 1000 µl of wine sample and 1000 µl of ultrapure water was vortexed and filtered (0.45 µm syringe filter) into an individual HPLC vial.

The vials were numbered and placed into the HPLC sequence programme with 10–12 samples completed each day.

ii) Instrumentation and operating conditions

Instrumentation consisted of an Agilent Technologies Agilent 1200 Series HPLC instrument (Waldbonn, Germany) with the following componentry: a G1322A vacuum degasser, G1311A quaternary pump, G1329A autosampler and G1321B 1260 Infinity fluorescence detector set at excitation and emission wavelengths of 330 nm and 440 nm, respectively. Separations were carried out on a Hypersil™ ODS 250 mm x 3 mm (5 µm particle size) column protected by Hypersil™ ODS 101 x 3 mm (5 µm particle size) drop-in guard cartridges both supplied by ThermoFisher Scientific (Lithuania).

Mobile phase A consisted of 95% 0.05 M sodium acetate buffer, pH 6.5 and 5% methanol. Mobile phase B consisted of methanol-acetonitrile 70-30. Both A and B were filtered under vacuum (0.22 µM nylon membrane). Separations were performed at 25 °C (flow rate 0.5 mL/min). The total run-time (including re-equilibration of the column) was 39 minutes. Table C shows the gradient programme.

Time (min)	%A	%B
0	97	3
4.5	95	5
10	81	19
16	73	27
20	58	42
25	52	48
32	40	60
35	97	3

Table C1 – Gradient programme for HPLC protocol

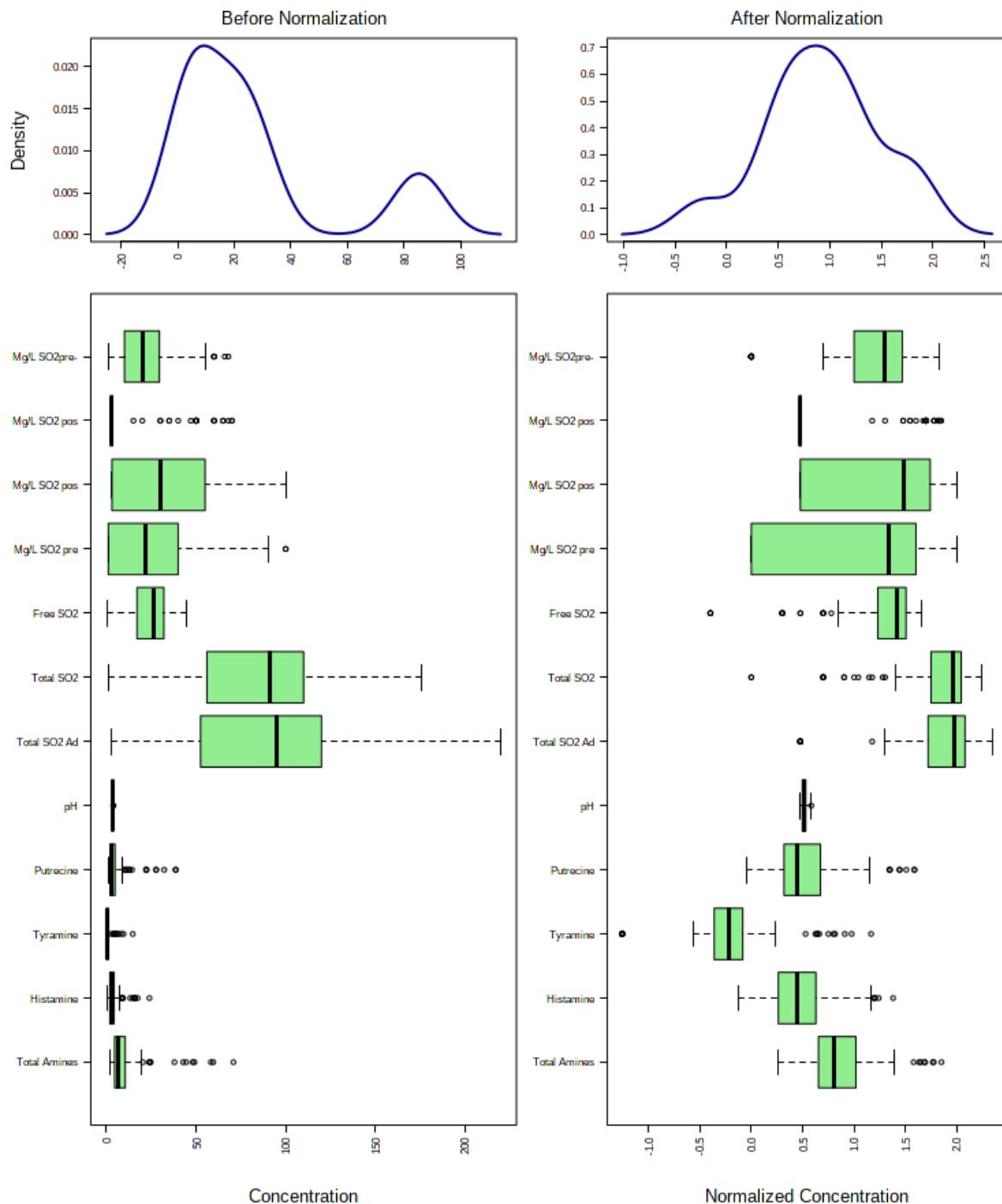
iii) Derivatisation of BAs

Table D outlines the in-loop derivatisation method for the HPLC protocol.

Step	Instrument action
1	Draw 1 µL from Vial 1
2	Draw 2 µL from Sample
3	Mix 5 times in air
4	Needle wash from Vial 2, 3 times
5	Draw 2 µL from Vial 3
6	Mix 15 times in air
7	Wait 3 mins
8	Draw 3 µL from Vial 4
9	Mix 5 times in air
10	Needle wash from Vial 5, 3 times
11	Inject
12	Wait 0.1 min
13	Valve bypass
Vial 1 0.2M Borate Buffer, pH 9.5M; Vial 2 10% Methanol; Vial 3 DR; Vial 4 Ultrapure Water; Vial 5 Ultrapure Water. Vials 1-5 filtered (0.45µm) into an amber HPLC bottle and numbered accordingly	

Table D2 – In-loop derivatisation injector program for HPLC protocol

Appendix 6: Data log-transformation



Appendix 7: Statistical analyses

Table E - One-way ANOVA with Fisher's LSD and adjusted p-values (FDR) for SG1 (n=100)

	f-value	p-value	FDR	Fisher's LSD
Total SO ₂ additions	332.85	1.39E-50	1.67E-49	Classic - Alternative; Alternative - Low-Add; Alternative - Zero-Add; Classic - Low-Add; Classic - Zero-Add; Low-Add - Zero-Add
Total SO ₂ (TSO ₂)	225.56	2.47E-43	1.48E-42	Classic - Alternative; Alternative - Low-Add; Alternative - Zero-Add; Classic - Low-Add; Classic - Zero-Add; Low-Add - Zero-Add
Free SO ₂ (FSO ₂)	82.694	1.65E-26	6.58E-26	Alternative - Low-Add; Alternative - Zero-Add; Classic - Low-Add; Classic - Zero-Add; Low-Add - Zero-Add
Total BAs (TBA)	42.011	1.99E-17	5.96E-17	Alternative - Classic; Low-Add - Alternative; Zero-Add - Alternative; Low-Add - Classic; Zero-Add - Classic; Zero-Add - Low-Add
Pre-bottling SO ₂ addition	39.725	8.84E-17	2.12E-16	Alternative - Zero-Add; Classic - Zero-Add; Low-Add - Zero-Add
Putrescine	36.418	8.36E-16	1.67E-15	Alternative - Classic; Low-Add - Alternative; Zero-Add - Alternative; Low-Add - Classic; Zero-Add - Classic; Zero-Add - Low-Add
Pre-AF SO ₂ addition	32.401	1.49E-14	2.55E-14	Classic - Alternative; Alternative - Low-Add; Alternative - Zero-Add; Classic - Low-Add; Classic - Zero-Add
Histamine	24.418	7.93E-12	1.19E-11	Alternative - Classic; Low-Add - Alternative; Zero-Add - Alternative; Low-Add - Classic; Zero-Add - Classic; Zero-Add - Low-Add
Tyramine	22.472	4.18E-11	5.57E-11	Alternative - Classic; Low-Add - Alternative; Zero-Add - Alternative; Low-Add - Classic; Zero-Add - Classic; Zero-Add - Low-Add
Post-AF SO ₂ addition	19.884	4.18E-10	5.01E-10	Classic - Alternative; Alternative - Low-Add; Alternative - Zero-Add; Classic - Low-Add; Classic - Zero-Add
pH	19.672	5.06E-10	5.53E-10	Low-Add - Alternative; Zero-Add - Alternative; Low-Add - Classic; Zero-Add - Classic
Post-MLF SO ₂ addition	7.3931	0.000165	0.000165	Alternative - Classic; Alternative - Low-Add; Alternative - Zero-Add

Table F - One-way ANOVA with Fisher's LSD and adjusted p-values (FDR) for SG2 (n=98)

	f-value	p-value	FDR	Fisher's LSD
Pre-AF SO ₂ addition	389.21	3.76E-57	3.01E-56	Stage 1 - Stage 2; Stage 1 - Stage 3; Stage 1 - Stage 4; Stage 1 - Zero added
Pre-bottling SO ₂ addition	34.051	1.74E-17	6.97E-17	Stage 1 - Zero added; Stage 2 - Zero added; Stage 3 - Zero added; Stage 4 - Zero added
Post-AF SO ₂ addition	32.869	4.53E-17	1.21E-16	Stage 1 - Stage 3; Stage 1 - Stage 4; Stage 1 - Zero added; Stage 2 - Stage 3; Stage 2 - Stage 4; Stage 2 - Zero added
Total BAs (TBA)	29.874	5.58E-16	1.12E-15	Stage 3 - Stage 1; Stage 4 - Stage 1; Zero added - Stage 1; Stage 4 - Stage 2; Zero added - Stage 2; Zero added - Stage 3; Zero added - Stage 4
Post-MLF SO ₂ addition	27.886	3.19E-15	5.11E-15	Stage 2 - Stage 1; Stage 3 - Stage 1; Stage 3 - Stage 2; Stage 2 - Stage 4; Stage 2 - Zero added; Stage 3 - Stage 4; Stage 3 - Zero added
Putrescine	23.38	2.14E-13	2.86E-13	Stage 3 - Stage 1; Stage 4 - Stage 1; Zero added - Stage 1; Stage 4 - Stage 2; Zero added - Stage 2; Zero added - Stage 3; Zero added - Stage 4
Histamine	21.413	1.53E-12	1.74E-12	Stage 3 - Stage 1; Stage 4 - Stage 1; Zero added - Stage 1; Stage 3 - Stage 2; Stage 4 - Stage 2; Zero added - Stage 2; Zero added - Stage 3; Zero added - Stage 4
Tyramine	18.546	3.10E-11	3.10E-11	Stage 3 - Stage 1; Stage 4 - Stage 1; Zero added - Stage 1; Stage 4 - Stage 2; Zero added - Stage 2; Zero added - Stage 3; Zero added - Stage 4

Table G – Pearson correlation co-efficient table (r values) for SG1

	pH	Tyramine	Histamine	Total BAs	Putrescine	Free SO ₂	Total SO ₂ addition	Total SO ₂
pH	1	0.4106	0.56872	0.64243	0.64779	-0.4514	-0.54369	-0.54274
Tyramine	0.4106	1	0.59862	0.69685	0.63691	-0.50328	-0.61336	-0.58018
Histamine	0.56872	0.59862	1	0.92848	0.7683	-0.41901	-0.58228	-0.53895
Total BAs	0.64243	0.69685	0.92848	1	0.9405	-0.56308	-0.68436	-0.6444
Putrescine	0.64779	0.63691	0.7683	0.9405	1	-0.60109	-0.68036	-0.63988
Free SO ₂	-0.4514	-0.50328	-0.41901	-0.56308	-0.60109	1	0.80431	0.81776
Total SO ₂ addition	-0.54369	-0.61336	-0.58228	-0.68436	-0.68036	0.80431	1	0.92512
Total SO ₂	-0.54274	-0.58018	-0.53895	-0.6444	-0.63988	0.81776	0.92512	1

Table H - Pearson correlation co-efficient table (r values) for SG2

	Pre-bottling addition	Pre-AF addition	Post-AF addition	Post-MLF addition	Tyramine	Histamine	Total BAs	Putrescine
Pre-bottling addition	1	0.30209	0.15898	0.11669	-0.317	-0.39959	-0.44748	-0.44094
Pre-AF addition	0.30209	1	0.60724	-0.29665	-0.55059	-0.59686	-0.6063	-0.54645
Post-AF addition	0.15898	0.60724	1	-0.30002	-0.51109	-0.47592	-0.50112	-0.46207
Post-MLF addition	0.11669	-0.29665	-0.30002	1	0.001948	0.10353	-0.00262	-0.07493
Tyramine	-0.317	-0.55059	-0.51109	0.001948	1	0.5909	0.69222	0.63351
Histamine	-0.39959	-0.59686	-0.47592	0.10353	0.5909	1	0.92558	0.76098
Total BAs	-0.44748	-0.6063	-0.50112	-0.00262	0.69222	0.92558	1	0.93924
Putrescine	-0.44094	-0.54645	-0.46207	-0.07493	0.63351	0.76098	0.93924	1

Appendix 8: Financial declaration

Samples purchased from commercial sources:

Approximately 80% of all samples were provided free of charge direct from winery producers, while the balance of samples was purchased by the author from commercial sources at retail prices.

Laboratory

All laboratory services as well as reagents, chemicals and instrumentation were provided free of charge by Plant & Food Research, Blenheim Campus (New Zealand). Specialist assistance was provided by Plant & Food Research staff during sample preparation and analyses.

Appendix 9: Research paper proposal

IMW Research Paper Proposal Submission Form			
Student ID	24860	Date of submission	22/11/20
RPP Version No	8	Name of Advisor	Julia Harding MW
Note: RPPs must be submitted via your Advisor to the IMW			
Proposed Title			
What is the Relationship Between the Use of Sulphur Dioxide and Biogenic Amine Levels in Wine?			
Research Questions: Define the subject of your Research Paper and specify the specific research questions you plan to pursue. (No more than 200 words)			
The focus of the research is to investigate biogenic amines (BAs) in wine and how their presence is affected by winemaking practices with particular reference to the amount and timing of Sulphur Dioxide (SO_2) additions.			
The research will address the following questions:			
<ol style="list-style-type: none">1) What is the relationship between the amount of SO_2 added during winemaking and the resulting levels of BAs in wine?2) What is the relationship between the timing of SO_2 additions during winemaking and the resulting levels of BAs in wine?3) Is there an argument for the creation of a new low-BA category of wine to aid those who suffer negative side effects from BAs, empowering them to make more confident wine purchasing decisions?			
Background and Context: Explain what is currently known about the topic and address why this topic requires/offers opportunities for further research. (No more than 200 words)			
Vast research exists on BAs in food and beverage science (including wine), yet limited understanding exists throughout the wine industry; from winemakers unaware of BAs' presence, cause and effects, through to gatekeepers/consumers/media with regard to their health implications. Current research demonstrates that BAs most likely cause the adverse reactions in			

wine consumers (termed ‘wine intolerance’) that are commonly attributed to sulphites or other wine components (not including alcohol), especially when over-indulgence in alcohol can be eliminated. Symptoms of BA toxicity/ingestion include nausea, headache, rhinoconjunctival symptoms, hyper- or hypotension, flushing, rashes and heart palpitations.¹

BAs are naturally occurring compounds derived from amino acids and are produced chiefly by decarboxylation. As a chemical group, they have many forms, with histamine the most commonly recognized and one of the most toxic to humans.² High BA levels are indicative of spoilage in many foods and are consequently used as a quality index (primarily in meat and fish) to signal their degree of freshness or deterioration,³ but are not customarily used to measure quality aspects in wine.

While BAs contribute to normal physiology, they can have toxic effects when consumed, particularly if the detoxifying enzymes are inhibited. Alcohol is one such potent inhibitor.⁴ These health and quality implications suggest wine-industry stakeholders should therefore be acutely aware of BAs, including winemaking pathways that contribute to their presence in wine. There has been little prior research into the relationship between BA levels and the use of SO₂. SO₂ impedes microbial activity and even small amounts are particularly effective against the most prolific BA-producing bacteria.⁵ The effective use of SO₂ and in particular the timing of addition(s) could therefore be a major tool in reducing BA levels by inhibiting the growth of BA-producing bacteria.

Furthermore, the current movement towards wines with low or zero SO₂ addition is seemingly at odds with BA management best practice, which highlights the absence of knowledge about this issue. It also underscores an apparent contradiction between SO₂ use in wine and the increasingly negative attitude toward it. The topic therefore requires exploration to clarify unfounded beliefs and shed light on the underlying public health issue of BAs in wine.

¹ Silla Santos., M.H., *Biogenic amines: their importance in foods*. Int. J. Food Microbiol. 1996, 29, 213-231; Taylor, S.L., *Histamine food poisoning, toxicology and clinical aspects*. Crit. Rev. Toxicol. 1986, 17, 91-128.

² Other forms of BAs are aliphatic (e.g., putrescine, cadaverine, spermidine and spermine), heterocyclic (e.g., histamine and tryptamine), or aromatic (e.g., tyramine and phenylethylamine).

³ Ruiz-Capillas, C., Herrero, A. M., *Impact of Biogenic Amines on Food Quality and Safety*. MDPI Foods. 2019, 8, 62.

⁴ Other potentiating co-factors that inhibit metabolism of BAs include aldehyde, other biogenic amines, certain amine oxidase-inhibiting drugs and pre-existing gastro-intestinal diseases. Costantini et al. *An Overview on Biogenic Amines in Wine*. MDPI Beverages. 2019, 5, 19.

⁵ Wells, A., Osborne, J. P., *Impact of acetaldehyde- and pyruvic acid-bound sulphur dioxide on wine lactic acid bacteria*. Letters in Applied Microbiology 2011, 54, 187–194.

Sources: Identify the nature of your source materials (official documents, books, articles, other studies, etc.) and give principle sources if appropriate. (No more than 150 words)

1) Published wine and food science and medical peer-reviewed papers, including but not limited to:

- Costantini et al. Review: An Overview on Biogenic Amines in Wine. MDPI Beverages. 2019, 5, 19.
- Smit, A.Y.Y.; Du Toit, W.J.J.; Du Toit, M. Biogenic amines in wine: Understanding the headache. S. Afr. J. Enol. Vitic. 2008, 29, 109–127.
- Granchi, L.; Romano, P.; Mangani, S.; Guerrini, S.; Vincenzini, M. Production of biogenic amines by wine microorganisms. Bulletin de l'O.I.V. 2005, 78, 595–609.
- Wells, A.; Osborne, J.P.; Impact of acetaldehyde- and pyruvic acid-bound sulphur dioxide on wine lactic acid bacteria. Letters in Applied Microbiology. 2011, 54, 187-194.
- Wantke, F., Manfred, G., Jarisch, R., The Red Wine Provocation Test: Intolerance to Histamine as a Model for Food Intolerance. Allergy Proc. 1994, Vol. 15, No. 1: 27-32.
- Jarisch, R., Wantke, F., Wine and Headache. Int Arch Allergy Immunol 1996; 110: 7-12.
- Maintz, L., Novak, N., Histamine and histamine intolerance. Am J Clin Nutr 2007; 85: 1185-96
- Silla-Santos, M. H. (1996) 'Biogenic amines: their importance in foods', *International Journal of Food Microbiology*, 29:213—31
- ten Brink, B., Damink, C., Joosten, H. M., Huis in't Veld, J. H. (1990) 'Occurrence and formation of biologically active amines in foods', *International Journal of Food Microbiology*, 11:73–84

2) Industry regulatory documentation including but not limited to:

- OIV; *OIV code of good vitivinicultural practices in order to minimise the presence of biogenic amines in vine-based products*. RESOLUTION OIV-CST 369-2011.

3) Individual sources (in the form of interview) including but not limited to:

- Dr. Benjamin McGettigan, MBChB BSc(Hons) FRACP FRCPA, Clinical Immunologist, Cambridge Specialist Centre, Perth, Western Australia. (Confirmed).

Research Methodology: Please detail how you will identify and gather the material or information necessary to answer the research question(s) and discuss what techniques you will use to analyse this information. (No more than 500 words)

This subject necessitates a two-part approach to research:

- 1) **Literature Review** - Undertake a review of existing research on wine intolerance, with a specific focus on BAs in wines and winemaking in order to determine and subsequently outline the different winemaking pathways that either increase or decrease BA levels in wine.
- 2) **New Zealand Wine BA Analysis** – Analyse 100 New Zealand (NZ) wines for BAs, using Reverse-Phase (RP) High-Pressure Liquid-Chromatography (HPLC) at an authorized research laboratory. The focus on NZ wines has two grounds: the researcher has ready access to NZ wines, and a BA study has not been conducted on NZ wines before.⁶

Based on the Literature Review to date, the researcher is confident a sample size of 100 will provide enough data to have a statistically acceptable confidence level. However, because the results are not known it is not possible to calculate the sample size or confidence levels using traditional statistical methods or calculators. This sample size is also a realistic number of samples to obtain, along with the concomitant technical information required taking into account the variables, listed below.

One variety, Sauvignon Blanc, will be used as this variety provides stylistic range and diverse winemaking practices (e.g. from 100% tank fermented to oaked, wild-fermented styles) and limits the variables of the study. The wines included will have different SO₂ regimes, from zero addition up to 160 mg/litre total SO₂. Wines will have been bottled within the past four years and will be selected to represent even distribution across the winemaking and SO₂ regime variables. The researcher is confident each winery will supply wine samples and the requisite information with the incentive of gaining information about their wine's BA levels and in the interests of research.

⁶ While there are many published papers that have studied BAs in wine, none have specifically looked at the relationship between BAs and SO₂ use and the timing of additions. Published papers have studied BAs with reference to geographical origin, variety, yeast species and malolactic conversion and the microbiology responsible, while wines used have originated primarily from Europe, with several papers using wines from the Americas, South Africa as well as one using Chinese wines. To the researcher's knowledge, no published paper has used Australian or New Zealand wines in a BA study.

Concentrations of histamine, tyramine and putrescine, identified as the three most relevant BAs in wine (in terms of toxicity),¹ will be quantified by HPLC fluorescence detection of o-phthaldialdehyde (OPA) derivatives for each wine sample by the lab, with results received, collated and analysed by the researcher.

The Literature Review has identified that the two macro-variables influencing BA levels are:

- a) Level of pre-cursor amino acids; and
- b) Microbial growth during winemaking.

The variables of the study are:

- 1) Total quantities of SO₂,
- 2) Timing of SO₂ additions and amount added:
 - i) zero addition
 - ii) addition immediately prior to fermentation
 - iii) immediately post-fermentation
 - iv) post-MLF (if applicable) / post-assemblage
 - v) pre-bottling.
- 3) Other relevant technical data that is necessary for BA assessment:
 - i) pH and its adjustment,
 - ii) Malolactic fermentation (full, partial or none),
 - iii) Time on skins,
 - iv) Lees contact and any stirring of lees.
- 3) From the analysis of this data, the researcher will endeavour to identify and discuss any patterns that exist between SO₂ regime and BA levels, as well as any patterns that emerge from the timing of SO₂ and resulting BA levels in the wine.

Feasibility issues:

- Laboratory testing fees in a commercial laboratory (estimated NZD\$35,000). However, research funding (wholly or co-funded) or an alternative arrangement with a laboratory capable of carrying out the analysis is being sought.⁷
- Obtaining wines that fit within the desired criteria. However, with the current fashion for wines with no or low SO₂, it is believed there will be an excess of valid samples.

⁷ Smit, A.Y.Y.; Du Toit, W.J.J.; Du Toit, M. *Biogenic amines in wine: Understanding the headache*. S. Afr. J. Enol. Vitic. 2008, 29, 109–127.

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The Literature Review has identified that the two macro-variables influencing BA levels are:

- c) Level of pre-cursor amino acids; and
- d) Microbial growth during winemaking.

The variables of the study are:

- 4) Total quantities of SO₂,
- 5) Timing of SO₂ additions and amount added:
 - i) zero addition
 - ii) addition immediately prior to fermentation
 - iii) immediately post-fermentation
 - iv) post-MLF (if applicable) / post-assemblage
 - v) pre-bottling.
- 6) Other relevant technical data that is necessary for BA assessment:
 - v) pH and its adjustment,
 - vi) Malolactic fermentation (full, partial or none),
 - vii) Time on skins,
 - viii) Lees contact and any stirring of lees.
- 4) From the analysis of this data, the researcher will endeavour to identify and discuss any patterns that exist between SO₂ regime and BA levels, as well as any patterns that emerge from the timing of SO₂ and resulting BA levels in the wine.

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⁸ The researcher acknowledges and agrees to be bound by the Institute's policy on research funding, including ensuring the research remains free from any commercial bearing or obligations, is free from any conflict of interest and that copyright remains exclusively with the Institute.

- Obtaining wines that fit within the desired criteria. However, with the current fashion for wines with no or low SO₂, it is believed there will be an excess of valid samples.

Potential to Contribute to the Body of Knowledge on Wine: Explain how this Research Paper will add to the current body of knowledge on this subject. (No more than 150 words)

As an industry we have a responsibility to address the issue of BAs in wine as a matter of public interest. Although it is widely acknowledged that some people suffer unpleasant side-effects from drinking wine (to varying degrees), there are widely disseminated misconceptions about the cause of those reactions. This is an opportunity to enable those who have avoided wine because of a reaction to a wine high in BAs to enjoy wine confidently, knowing they will not face the ill-effects of BA toxicity.

This research will elucidate and communicate the issue of BA levels in wine and the varying winemaking pathways that affect BA levels in order to assist the industry in producing wines lower in BAs.

The focus on the use of SO₂ in relation to BA levels has never been researched in isolation before, nor has any research to date studied BAs in NZ wine. It will therefore provide new knowledge which will better the industry and ultimately benefit the Institute.

Proposed Time Schedule/Programme: This section should layout the time schedule for the research, analysis and write-up of the Research Paper and should indicate approximate dates with key deliverables. *Dates of submission to both Advisors and the IMW must be those specified by the IMW.*

November/December 2019 – collection of samples and technical information for BA testing.

January/February 2020 – commence writing Introduction and Literature Review.

– *Disruption due to Covid-19 lockdowns* –

May/June 2020 – RP HPLC analysis of wines at research laboratory.

June/July 2020 – collation and analysis of raw data from RP HPLC analysis.

August/September 2020 – completion of Literature Review, Methods and Appendices. Data analysis completed.

September/October 2020 – completion of Results and Conclusions write-up.October 2020 - confirmation of RP submission by 9/10. First draft completed by 19/10, proof-reading by external entity. Completed draft with bibliography ready by 30/10.

November 2020 – Submission of final draft RP to Advisor by 6/11 and any revisions to RPP made and submitted to Institute. Revisions to final draft RP if necessary.

December 2020 – Final submission of finished RP to Institute (via Advisor) by 14/12.