

Research Article

Fate of Fumonisin B2 (FB2) during Vinification and Wine Storage: Fining Agents for the Removal of FB2 in Wine

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Background and Aims. Fumonisin is a group of mycotoxins produced by some isolates of black aspergilli. This study examines the dynamics of fumonisins during winemaking and storage and the role of fining agents in the removal of FB2, the most prevalent fumonisin produced by black aspergilli. **Methods and Results.** Chardonnay grapes were inoculated with a fumonisin-producing strain of *Aspergillus welwitschiae* and fumonisins were quantified at different stages of vinification. The stability of fumonisins was studied at 13 and 25°C using Chardonnay wine spiked with FB2. Activated charcoal, calcium bentonite, yeast hulls, and a nonallergenic polysaccharide of nonanimal origin (NO (OX)) were evaluated as fining agents for FB2 removal from wine. Fumonisin was quantified by LC-MS/MS. During vinification most of the fumonisins were removed with the solids, with only 3% of the fumonisins found in the grapes being transferred to the wine. Fumonisin FB2 was stable in white wine for at least 4 months, but was reduced by 42% after 8 months at 25°C. Activated charcoal and bentonite were able to remove more than 80% of FB2 in white wine. **Conclusions.** Vinification can lead to the partial removal of fumonisins. The use of fining agents and wine storage leads to further reduction. **Significance of the Study.** This study demonstrates the fate of fumonisins during vinification and wine storage and the potential use of fining agents for amelioration of fumonisin contamination.

1. Introduction

Mycotoxin occurrence in wine has been a concern since the first detection of ochratoxin A (OTA), a potential human carcinogenic compound, in wine in 1996 [1]. Since then, studies have been carried out worldwide to identify the occurrence of OTA in wine, and consequently, OTA management strategies have been identified to mitigate the risk of the formation of this toxin in wine [2–4]. Another group of mycotoxins and fumonisins, which are also known to be potentially carcinogenic [5], gained significant attention after they were identified in grapes and grape products worldwide [6–11]. Fumonisin B2 (FB2) is the most significant fumonisin with regards to grape and wine production and was first detected in must samples from Italy [6], and then, in wine [7, 12]. A study involving 77 wines from 13 different countries including Australia, found that 18 of these wines were contaminated with fumonisin B2, and the amount detected varied

depending on the wine style and the country of origin [12]. Regulations are in place that specifies the amount of FB2 and two other fumonisins (FB1 and FB3) permitted in several food commodities [13]. Wine is not included in this list, however, possibly due to the low concentration found in wine (0.4–25 µg/L). Although the amount of FB2 produced by aspergilli in grapes appeared to be small (0.1–7.8 mg/kg) [6, 8, 10] and may not pose a great risk, this could largely be affected by many factors including strains of black aspergilli and eco-physiological conditions [14, 15]. Given that black aspergilli commonly occur in grapes close to harvest [3, 16, 17], there is a potential risk that wine made from infected grapes could be contaminated, unless infected berries are removed before vinification. Under commercial conditions, physical removal of infected grapes is not always feasible, especially in countries where grapes are machine harvested as opposed to hand harvested. Options for the removal of FB2 from wine need to be investigated.

Mycotoxin contamination of wine grapes can be at least partially removed during vinification. A large portion of the mycotoxin found in grapes is removed with the solids, and only a small portion is passed into the final wine [18–23]. Extensive research conducted on OTA revealed that the amount of OTA removed during vinification is affected by many factors including grape cultivar, wine type, yeast, and use of fining agents [2]. Moreover, storing wine contaminated with OTA for a period of time resulted in a reduced concentration of OTA [24]. Little is known, however, about the fate of FB2 during vinification and wine storage and if fumonisins behave in a similar way to OTA during the winemaking process. Studies conducted on fumonisin contamination in wine have largely involved muscadine grapes, *Vitis rotundifolia* rather than the European grapevine, *V. vinifera*.

Lewis [21] studied the role of vinification in the removal of fumonisins using must samples from *V. rotundifolia* grapes (cvs Carlos and Noble) spiked with FB2. Interestingly, 70–80% of FB2 was removed with the solids, depending on the initial concentration of FB2 in the must samples and the grape cultivar. Additionally, the authors reported that the cold stabilisation of wine samples led to a minor reduction of FB2. Further studies with *V. vinifera* grape cultivars are required to understand the underlying factors that influence the removal of fumonisins during vinification.

Fumonisin is amenable to hydrolysis in some food commodities and under certain food processing conditions [21, 25, 26]. Lewis [21] identified hydrolysed products of FB2 after storage of muscadine grape juice at a range of temperatures (–20–20°C) over an 8 month period. Storage is a general requirement of any wine production process; however, no studies have been carried out to investigate the potential hydrolysis of FB2 during wine storage. Although the toxicity of the hydrolysed products of fumonisins is yet to be fully evaluated [27], some studies have demonstrated that these products could also be as cytotoxic as the parent compounds [28]. Therefore, the presence of the hydrolysis products of FB2 needs to be investigated in wine after storage to identify the actual health risk posed by fumonisins for wine consumers.

The elimination of mycotoxins, particularly OTA, in wine has been approached in several instances using fining agents that are regularly used in wine clarification [29–33]. The removal of fumonisins from a model wine solution and a red wine (cv. Cabernet Sauvignon) has been studied using polymeric compounds [29]. However, application of these agents for the removal of fumonisins from white wine is yet to be explored. Wineries use fining agents for a range of purposes [34, 35], and therefore, exploration of these agents for the removal of the fumonisins within the dosage recommended by the wine industry may be desirable for commercial usage.

Our research investigated the change in fumonisin concentration at the different stages of Chardonnay wine vinification to determine the amount of fumonisins transferred into wine from grapes infected with a fumonisin-producing *A. welwitschiae* isolate. The effect of fining agents, within the dosage recommended for clarification of white

wine, was investigated for their ability to remove fumonisins from wine. Additionally, the influence of wine storage and handling conditions on the stability of fumonisins was also studied to determine the impact of these factors on the elimination of fumonisins in white wine.

2. Materials and Methods

2.1. Chemicals and Biochemicals. Yeast extract powder and K_2HPO_4 , KCl, $NaNO_3$, $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot H_2O$, $CuSO_4$, and $ZnSO_4$ for preparation of Czapek yeast extract agar (CYA) medium were purchased from Sigma–Aldrich (Castle Hill, NSW, Australia). Solvents used were HPLC grade and included acetonitrile (Chem-Supply, Gillman, SA, Australia) and ultrapure water (Millipore, North Ryde, NSW, Australia). Formic acid (LC/MS grade, LiChropur, 98–100%, Sigma–Aldrich) was used to acidify the mobile phase. Fumonisin standards (FB1, FB2, and FB3) (Sigma–Aldrich) were initially used for method development, and FB2 was used in preparation of calibration standards. Fining agents, activated charcoal (AC) (OtaClean, a granular form of carbon), calcium bentonite, polyvinylpyrrolidone (PVPP), NO (OX) (nonallergenic polysaccharide of non-animal origin) was purchased from (Winequip, Reservoir, Vic., Australia). Yeast hulls (Reskue) were purchased from Lallemand, Edwardstown, SA, Australia.

2.2. Fungal Isolate. *Aspergillus welwitschiae* (YO 1.2) was isolated from *V. vinifera* (cv. Nebbiolo) grapes from a vineyard in southern NSW and maintained on CYA as previously described [15]. This isolate was previously identified as a high producer of fumonisins when cultured in synthetic grape juice medium. Approximately 20 mg of fumonisin per kilogram of synthetic grape juice medium were formed after 7 days of incubation in the dark at 25°C [9].

2.3. Preparation of Spore Suspensions. *Aspergillus welwitschiae* (YO 1.2) was grown on CYA medium at 25°C in the dark for 7 days. Spores were harvested by gently scraping the cultures with a sterile spreader (Thermo Fisher Scientific, Thebarton, SA, Australia) after flooding the cultures with sterile distilled water containing 0.5 g/L Tween 80 (Merck Life Science, Bayswater, Vic., Australia). The resulting suspension was vortexed thoroughly to break up the spore clumps and filtered using double-layered Mira cloth (Merck Life Science) to remove mycelial fragments. The spores were enumerated with a haemocytometer, and the concentration of the spore suspension was adjusted to 1×10^7 spores/mL using sterile distilled water.

2.4. Inoculation and Incubation of Grape Bunches for Winemaking. *Vitis vinifera* (cv. Chardonnay) grape bunches (average 12.2 Baume) were collected from an apparently disease-free vineyard (as determined by visual inspection) in southern NSW (Gundagai, Australia). Bunches were visually inspected, and berries with signs of

infection or damage (cracks and sunburn) were removed. Bunches were surface sterilised with sodium hypochlorite (1.0% v/v, plus 0.05% v/v Tween 80) for 2 min and rinsed three times with sterile distilled water. Approximately 30 berries from each bunch were wounded by puncturing with a sterile needle (26 G). Whole bunches were then dipped in a beaker containing 500 mL of the *A. welwitschiae* (YO 1.2) spore suspension, and the excess suspension was allowed to drain from the bunches prior to placing them inside pre-sterilised boxes. Bunches were arranged on a sterilised wire rack and placed in a disinfected plastic container such that bunches did not make contact with each other. To achieve high humidity, 300 mL of sterilised distilled water was added into each container prior to sealing the boxes. Bunches were placed in a growth chamber in the dark at 30°C to facilitate initial infection of the berries. After 3 days, the temperature was reduced to 25°C, and the incubation was continued for an additional 4 days to allow for further growth of the fungus. Control bunches (approximately 2 kg/replicate) were punctured in a similar manner, dipped in sterile distilled water, and incubated under the same conditions as described above for the inoculated bunches.

2.5. Constituting Disease Severity Levels. From the inoculated bunches, approximately 30 bunches were selected with similar disease severity based on visual appearance. Three infection levels: low, medium, and high, which corresponded to 25, 50, and 100% of the bunch showing signs of *Aspergillus* infection were artificially constituted by mixing healthy bunches with infected bunches on a mass-to-mass basis. A fourth batch of grapes that were uninoculated served as a control sample. Each sample consisted of approximately 2 kg of bunches, with three replicates for each of the four disease severity levels.

2.6. Vinification. The four batches of grapes were vinified at the Charles Sturt University Experimental Winery (Wagga Wagga, NSW). To extract juice, grape samples from each severity level were pressed using a hydraulic press (Stow, Bristol, England), and the juice was collected separately into 1 L Schott bottles. Each sample was pressed three times with similar pressure (200 kPa). The control samples were pressed first followed by the rest of the samples, starting with the grapes with the lowest severity of infection. The chamber of the press was thoroughly washed with distilled water between pressings of each severity level. Marc (skins, stems, and seeds) were separately collected for each severity level and stored at -20°C prior to extraction of fumonisins.

Potassium metabisulfite (PMS) (Winequip, Reservoir, Vic., Australia) was added to generate 60 mg/L total SO₂ in juice. The juice samples were overlaid with N₂ gas and kept in a cold room at 4°C for 24 h to precipitate solids. Total soluble solids were measured with a DMA 35 N Anton Paar portable density meter (Graz, Austria). The pH of the samples was adjusted to 3.3 by adding tartaric acid. The juice samples were inoculated with *Saccharomyces cerevisiae* (DV10; Eaton, Dublin, Ireland) at a rate equivalent to 0.25 g of dry yeast/L of juice. Fermentation was carried out at

16 ± 2°C, and the TSS and temperature were measured daily to monitor the fermentation. Diammonium phosphate (DAP) (0.3 g/L of juice) was added during fermentation to provide nutrients for yeast growth as required. When TSS reduced to below 0.1%, the wine was racked, and PMS was added to generate 60 mg/L total SO₂ in the juice to prevent oxidation. A second racking was performed as required, and PMS was added to bring the free SO₂ to 25 mg/L to ensure that the molecular SO₂ was approximately 0.5 mg/L. The bottles were overlaid with CO₂ and held at 4°C for cold stabilisation for 30 days. At this time, wine samples were analysed for pH, TA, and the concentrations of ethanol, free SO₂, and total SO₂ (Table S1).

2.7. Sampling. After pressing, marc was separately collected for each replicate, from all disease severity levels, to estimate the amount of fumonisins from a kilogram fresh mass of grapes, and to estimate the amount of fumonisins removed during solids separation. Similarly, juice or wine samples were collected after pressing, after fermentation, and after cold stabilisation. Samples of lees were also retained after racking to estimate how much fumonisin was removed with the lees. All samples, including wine were stored at -20°C in polypropylene centrifuge tubes with screw caps (Thermo Fisher Scientific) before analysis of fumonisins. The wine production and sampling process followed in this study is outlined in Figure S1.

2.8. Effect of Fining Agents in Removal of FB2

2.8.1. Wine Samples. A commercial bottle of Australian Chardonnay wine (Yarra Valley, Australia, 2018 vintage, pH 3.2, ethanol 12.0%) without detectable fumonisins (as tested before spiking) was spiked with FB2. The concentration of FB2 in the wine was adjusted to 1 mg/L. The spiked wine sample was aliquoted into 20 mL glass vials (5 mL wine per vial) and stored at -20°C until required. Wine samples were kept at 4°C overnight prior to fining agents being added. Upon addition of the fining agents, the samples were incubated at 20°C in the dark.

2.8.2. Preliminary Screening. Preliminary screening of the fining agents was conducted to identify the most effective agents for the removal of FB2 in white wine and to identify the required contact time. The fining agents, calcium bentonite and PVPP were used at a maximum concentration of 1 and 0.8 mg/L, respectively, as recommended by the Australian Wine Research Institute (AWRI) for clarification of white wine [36]. Activated charcoal was initially tested at a concentration of 1 mg/L and of 2 mg/L, which is the maximum recommended concentration for the clarification of white wine by the AWRI. Each concentration resulted in approximately 100% removal of FB2; hence, the use of 1 mg/L activated charcoal in the latter experiments. A relatively new fining agent, NO (OX) was used at 0.6 mg/L, which is the maximum concentration recommended for white wine by the manufacturer (Winequip). For yeast hulls

(Lallemand), there is no maximum concentration recommended by the AWRI, and therefore, a concentration of 1 g/L was used according to Zara et al. [37].

To determine the contact time required to remove FB2 from white wine, each fining agent was allowed to have contact with FB2 for 24 and 48 h.

2.9. Effect of the Concentration of Fining Agents in the Removal of FB2. To assess the effect of concentration of fining agents on FB2 removal, activated charcoal and calcium bentonite, which showed a capacity to remove more than 50% FB2, were further tested with two lower concentration values together with the concentration values tested during preliminary screening. The contact time was selected based on the results of the preliminary screening. All samples were incubated in the dark at 20°C with agitation (70 rpm) in a reciprocating shaking incubator (Thermo Fisher Scientific). Control samples without fining agents were subjected to the same experimental conditions. Samples were prepared in triplicate. At the end of the allocated incubation time, the samples were filtered through 0.2 µm RC syringe filters (Phenomenex, Lane Cove West, NSW, Australia) and analysed by LC-MS/MS as described in the following.

2.10. Stability of Fumonisins in White Wine during Storage. To study the stability of fumonisins during wine storage, 200 mL of Chardonnay wine (commercial) (pH 3.2, ethanol 12.0%) was spiked with FB2 (0.1 mg/L), aliquoted into 2 mL HPLC vials, and incubated at 13 ± 2 and 25 ± 1°C.

Vials were collected in duplicate every month for 6 months and stored at -20°C for later analysis. The experiment was continued for 8 months, and at the end of the study, samples were analysed for FB2 and hydrolysed FB2 (HFB2) using LC-MS/MS as described in the following. Quantification of FB2 was conducted using a matrix match calibration curve prepared as described below. The unavailability of a commercial HFB2 standard was overcome by hydrolysing a FB2 pure standard in 1 mol/L KOH according to the method described by Pagliuca et al. [38]. Figure 1 shows the chemical structures for FB2 and FB4 (a) and HFB2 (b). Retention time and MS/MS parameters were assigned with HFB2 to verify if HFB2 was detectable in the stored wines.

2.11. Sample Extraction and LC-MS/MS Analysis of Fumonisins. Fumonisins were extracted from 1 mL of each juice sample with a 4 mL mixture of methanol/H₂O 3/1 (v/v) by shaking at 120 rpm in a rotary shaker for 1 h. Fumonisins were extracted from lees and marc in a manner similar to juice, but using 5 mL of methanol as the extraction solvent, and extracting 1 g of marc or lees. All samples were centrifuged, then filtered using regenerated cellulose (RC) 0.2 µm syringe filters (Phenomenex) directly into HPLC vials and stored at -20°C until LC-MS/MS analysis. An Agilent 1260 binary pump was used with an Agilent 1200 autosampler in series, a degasser, and an Agilent 6470 triple quadrupole tandem mass spectrometer (MS/MS) detector

(Agilent Technologies, Palo Alto, CA, USA). A Gemini C6-phenyl column (Phenomenex; 50 × 2 mm, 3 µm, 110 Å) fitted with a security guard system was maintained at 25°C. A 5 µL injection volume was used for all standards and samples. Reverse-phase separation was obtained using a gradient of solvent A (ultrapure water + 20 mmol/L formic acid) and solvent B (acetonitrile + 20 mmol/L formic acid). The column was equilibrated for 25 min prior to analysis. The gradient was as follows: (i) held at 20% B for 3 min; (ii) linear from 20 to 55% B from 3 to 5 min; (iii) linear from 55 to 100% B from 5 to 6 min; (iv) held at 100% B from 6 to 10 min before returning to; (v) 20% B from 10 to 25 min. A constant flow rate of 0.3 mL/min was maintained throughout the analysis. The mass spectrometer was operated in positive mode (ESI⁺) at a source flow of 700 L/h nitrogen at 350°C. All the analyses were conducted using multiple reactions monitoring (MRM) mode. Identification of the analytes was approached using molecular mass, fragmentation pattern, and comparison of the RT of fumonisin standards where possible. Two product ions were monitored for FB2 and FB4 at the following MRM transitions: FB2 quantifier *m/z* 706 < 336, fragmentor 50 V, collision 25 V, dwell time 100 ms, qualifier *m/z* 706 < 512, fragmentor 50 V, collision 40 V, dwell time 50 ms; FB4 quantifier *m/z* 690 < 320, fragmentor 50 V, collision 25 V, dwell time 100 ms, qualifier *m/z* 690 < 514, fragmentor 50 V, collision 40 V, dwell time 100 ms. Fumonisins were quantified using matrix match calibration curves prepared separately for juice, wine, lees and marc. In each case, sample matrix with no detectable amount of fumonisins was extracted with methanol similar to the sample extraction procedure followed for each matrix as described above. For wine, direct addition of fumonisin was conducted followed by filtration. Two calibration curves were prepared for wine and juice, concentration ranged from 0.0001–0.1 and 0.1–0.5 mg/L and, 0.0001–0.1 mg/L and 0.1–0.8 mg/L, respectively. The concentration of the calibration curves for lees and marc ranged from 0.0025–0.02 and 0.001–2 mg/L, respectively. All calibration curves showed good linearity, with the coefficient of determination (*R*²) better than 0.98 for all analyses. Where necessary, quantification of fumonisin B4 was conducted using the same calibration curve used for FB2, as FB4 standards were not commercially available. The concentration of FB4 is therefore reported as FB2 equivalents. The recovery values for each matrix were calculated by spiking each sample type with 0.1 and 0.5 mg/L of FB2 prior to the extraction of the sample. The recovery for juice, lees, and marc was 92% (RSD~7%), 42% (RSD~7%), and 30% (RSD~10), respectively. The limit of detection (LOD) for FB2 was calculated using the equation $LOD = (3.3 * SD/b)$ (SD of the noise for ten blank measurements, b-slope of the calibration curve) and the limit of quantification (LOQ) was calculated using the equation, $LOQ = (103 * SD/b)$ [39]. Table 1 shows the LOD and LOQ calculated for each matrix.

2.12. Statistical Analysis. All statistical analyses were performed with SPSS (IBM SPSS Statistics v. 20, 2015, IBM Corporation, Sydney, NSW, Australia). The data were

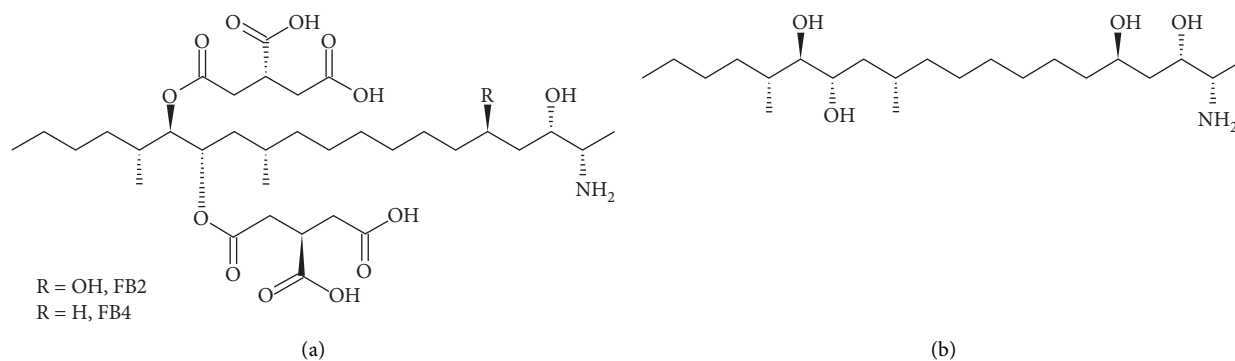


FIGURE 1: Chemical structures of (a) fumonisin B2 and B4, and of (b) hydrolysed fumonisin B2.

TABLE 1: Limit of detection and limit of quantification for fumonisin B2 in juice, wine, lees and marc.

| Matrix | LOD ($\mu\text{g}/\text{kg}$) | LOQ ($\mu\text{g}/\text{kg}$) |
|--------|---------------------------------|---------------------------------|
| Juice | 0.16 | 0.48 |
| Wine | 0.07 | 0.25 |
| Lees | 0.25 | 0.90 |
| Marc | 0.25 | 0.95 |

LOD, limit of detection; LOQ, limit of quantification.

examined by ANOVA when they were amenable to that statistical analysis. The comparison of the means was conducted using the least significant difference (LSD) test. LC-MS/MS data processing and integration of peak areas were performed using Agilent Mass Hunter qualitative software (B.07.00).

3. Results

3.1. Effect of *A. Welwitschiae* Infection on TSS and TA of Chardonnay Grapes and Juice. In artificially inoculated bunches, *A. welwitschiae* grew from the site of wounding on the berries and produced browning of the berry surface as the fungus grew and eventually sporulated. Five days after inoculation, some berries became detached from the pedicel. Inoculated berries with no wounding remained uninfected. In addition to the browning of the berries, the infection caused berry softening and a reduction in TSS that decreased to 10.2 ± 0.4 in inoculated grapes. The TSS did not change in the control uninfected grapes and remained at 12.2 ± 0.4 Be 7 days postinoculation. Furthermore, the TA was higher in the juice from infected grapes (9.6 ± 0.2 g/L) than the control grapes (4.1 ± 0.1 g/L) 7 days postinoculation.

3.2. Fumonisin Concentration of Infected Grapes. The average concentration of fumonisins (FB2 and FB4) from a kilogram of grapes was calculated for each infection level (low, medium, and high) and the control uninoculated grapes by combining the average amounts found in the marc and juice samples. The amount of FB4 found in all samples was relatively low and ranged from 20 to 40% of the total amount of fumonisins found in the grape samples (Table 2).

TABLE 2: Mean fumonisin concentration in grape samples used for winemaking as calculated using the amount present in juice and marc.

| Infection level of grapes [†] | Mean fumonisin concentration in grapes ($\mu\text{g}/\text{kg}$) | |
|--|--|------------------|
| | FB2 | FB4 |
| Control | n.d. | n.d. |
| Low | 118 (± 20) | 99 (± 10) |
| Medium | 166 (± 8) | 122 (± 20) |
| High | 465 (± 49) | 127 (± 16) |

Mean fumonisin concentration in grapes was calculated by using the fumonisin concentration found in juice and marc from a kilogram of grapes (mean \pm SE, $n = 3$); the concentration of FB4 is based on FB2 equivalents. n.d., not detected. [†]Control, 0% infected; low, 25% infected; medium, 50% infected; high, 100% infected.

3.3. Fumonisin during Vinification. Figure 2 shows the fate of fumonisins during white wine production. The concentration of fumonisins was calculated in each matrix (marc, juice, lees, and wine) and expressed as the amount of total fumonisins per kilogram fresh mass of grapes by taking the mass of each matrix into consideration. In marc samples, FB4 was detected from all infection levels of infection in grapes; however, in other samples (juice and lees), FB4 was detected only from the medium and high infection levels. When it was quantifiable, the amount of FB4 was about 15 to 40% of the total amount of fumonisins.

A large portion of fumonisin was removed with the marc, which was separated after the grapes were pressed. The average proportion of fumonisins (calculated from values of the low, medium, and high infection levels of grapes) removed with the marc was calculated to be $86 \pm 4\%$ (82–90%). Juice samples contained an average of $14 \pm 4\%$ of fumonisins that were found in grapes; however, wine samples after fermentation contained only $3 \pm 0.3\%$ of the fumonisins that were found in the grapes prior to vinification.

Fumonisin was detected in juice and wine from all infection levels of grapes used for winemaking and were absent from the uninoculated grapes. As observed with marc, the highest concentration of fumonisins was detected from the high infection level of grapes and the lowest was observed for low infection level of grapes. Wine after fermentation, and after cold stabilisation (after 30 days of storage at 4°C) from control grapes with no signs of *Aspergillus* infection had

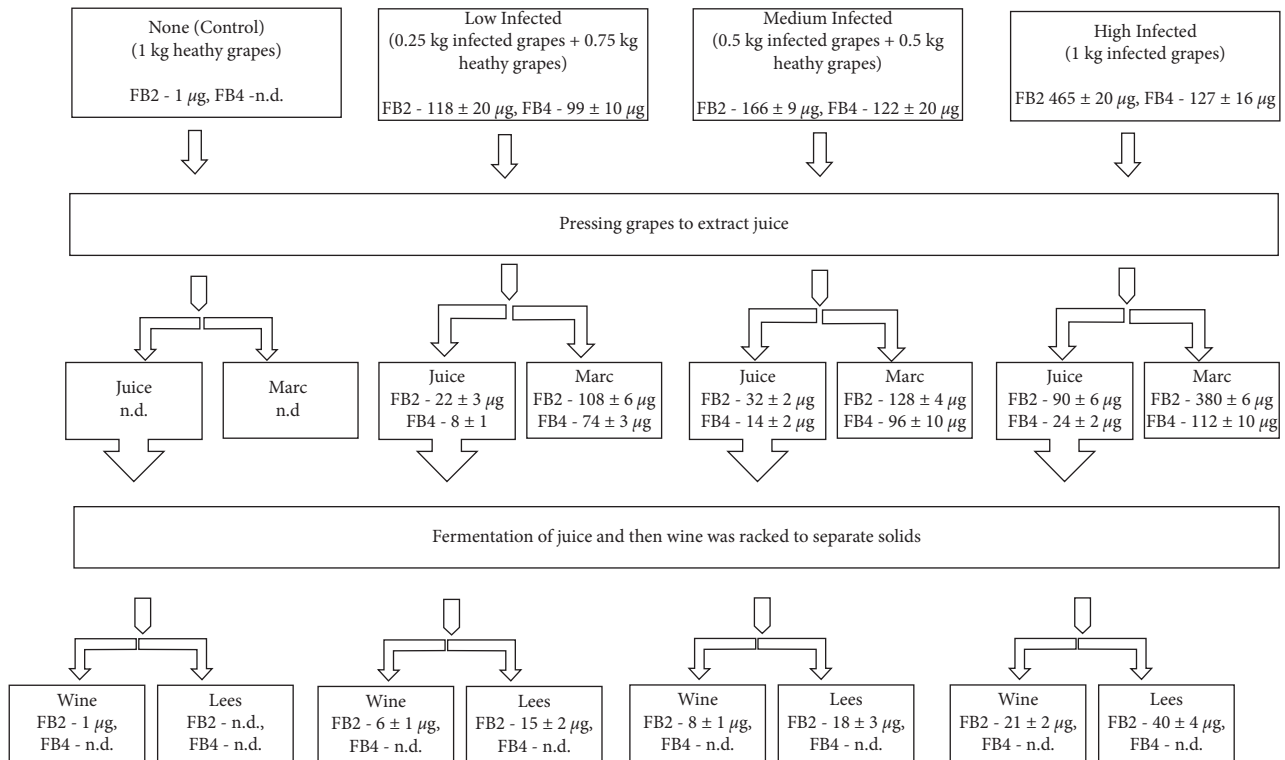


FIGURE 2: Fate of fumonisins during vinification of chardonnay grapes showing the concentration of fumonisins (FB2 and FB4) present in marc, juice, wine, and lees from a kilogram of grapes (mean \pm SE, $n = 3$). The fumonisin values have been calculated by taking the mass of each matrix into consideration. n.d., not detected.

a small amount (1 µg/kg) of fumonisins (Figure 2). Juice sample data from all infection levels revealed that approximately $20 \pm 5\%$ of the fumonisins found in the juice transferred into the wine. The lees sample, however, separated after fermentation, contained $45 \pm 15\%$ of the fumonisins found in the juice. The fumonisin concentration in wine was analysed after cold stabilisation at 4°C for 30 days. There was no significant difference in the amount of fumonisin detected in the wine samples before and after cold stabilisation. Overall, the results showed that the separation of solids (marc and lees) leads to the removal of approximately 90% of the fumonisins found in grapes, and only about 3% is passed into the wine. Approximately 7% of the total fumonisins found in grapes were not accounted for in the collective amounts observed in juice, marc, wine, and lees.

3.4. Effect of Fining Agents on Removal of FB2 from White Wine

3.4.1. Preliminary Screening. Activated charcoal was the most effective agent for the removal of FB2 from white wine, with approximately 100% of FB2 removed at both contact times. The second most effective agent was calcium bentonite, with 71 and 81% of FB2 removed in samples treated for 24 and 48 h, respectively. Yeast hulls removed 21 and 35% of FB2 after 24 and 48 h contact times, respectively (Table 3).

Among the five fining agents tested, activated charcoal and calcium bentonite were the most effective in removing FB2 from white wine, and were therefore selected for further

TABLE 3: Effects of fining agents on the removal of 1000 µg/L FB2 from white wine after 24 and 48 h treatment.

| Fining agent | Concentration of FB2 (µg/L) | |
|--------------------|-----------------------------|-------------------------|
| | 24 h | 48 h |
| Activated charcoal | 4 ± 0.7 _a | 3 ± 0.2 _a |
| Calcium bentonite | 292 ± 15 _a | 194 ± 3 _b |
| NO (OX) | 896 ± 3 _a | 1005 ± 18 _b |
| PVPP | 760 ± 5 _a | 830 ± 6 _b |
| Yeast hulls | 786 ± 12 _a | 648 ± 18 _b |
| Control | 1090 ± 1.5 _a | 1093 ± 1.8 _a |

Mean \pm SE ($n = 3$); same letters horizontally indicate no statistical difference as analysed using least significant difference test (LSD) ($P = 0.05$).

investigation at concentration of 0.25 g/L, 0.5 g/L with a contact time of 48 h. Polyvinylpolypyrrolidone and NO (OX) were different to the other agents in that they achieved maximum proportions of removal 26 and 10%, respectively, after 24 h contact time. The maximum amount of fumonisin removed with yeast hulls was 35%, which was achieved after 48 h of contact time.

3.4.2. Effect of Bentonite and Activated Charcoal in Removal of FB2 in Chardonnay Wine. Both calcium bentonite and activated charcoal were effective in removing FB2 in white wine, as observed in the preliminary screening. For calcium bentonite, the proportion of FB2 removal significantly increased with increasing concentration of the fining agent. For activated charcoal, however, this increment was only

significantly different between samples treated with a low (0.25 g/L) and medium concentration (0.5 g/L) of fining agent. Overall, the application of activated charcoal at eight times less concentration (0.25 g/L) than the maximum dose recommended by the AWRI (2 g/L) for clarification of wine was sufficient in removing 80% of FB2. To achieve similar results with calcium bentonite, the maximum concentration of 1 g/L recommended by the AWRI was required (Figure 3).

3.5. Stability of Fumonisin in Wine. The concentration of FB2 in wine remained the same for up to 5 months in wine samples incubated at 13°C. At 25°C, however, the FB2 concentration gradually diminished for the first 5 months of storage, and thereafter it decreased to 0.08 mg/L after 8 months (Figure 4). The overall decrease in FB2 concentration was about 14 and 42% for the wine samples incubated at 13 and 25°C, respectively, for 8 months of storage.

The wine samples that were incubated at 25°C were analysed for hydrolysis products by LC-MRM-MS. This analysis was undertaken in an attempt to identify if indeed it was hydrolysis that led to the reduction of FB2 during the incubation time. A small peak related to HFB2 was detected in wine samples stored at 25°C for 8 months period; however, this was below the LOQ (Figure 5).

4. Discussion

Infection of Chardonnay grapes by *A. welwitschiae* resulted in physical changes such as browning and softening of the berries. Juice samples from *Aspergillus*-infected grapes had lower TSS and elevated TA compared to that of uninfected grapes. Lower TSS from infected grapes may be due to the depletion of the nutrients by the fungus during growth and metabolism on the berries [17]. In contrast, higher TSS has been reported for juice samples from Chardonnay grapes infected with *A. carbonarius*, potentially due to berry shrinkage as a result of the infection [24]. In the current study, however, *A. welwitschiae* infection did not cause berry shrinkage (assessed visually) over the incubation period of 7 days. *Aspergillus* spp. are known to produce organic acids, such as citric and gluconic acid, during their metabolism [17, 24, 40], and the higher TA in juice samples from inoculated grapes could be related to the presence of these acids.

The most notable change in the amount of fumonisins during white vinification occurred when grapes were pressed, where 86% (average calculated for three infection levels of grapes) of fumonisins found in grapes were removed with the marc. Previous investigations related to the effect of white vinification on the removal of OTA have revealed that about 80% of OTA is removed with the marc and 20% is passed into the juice [20]. Conversely, Lewis [21] investigated the fate of FB2 using must samples from nonvinifera grapes (*V. rotundifolia*, cvs Carlos and Noble) spiked with FB2, and the results showed that there was an increase in FB2 in juice samples compared to that in must samples. This was presumed to be due to the natural contamination of the grape samples with fumonisins. Lewis [21]

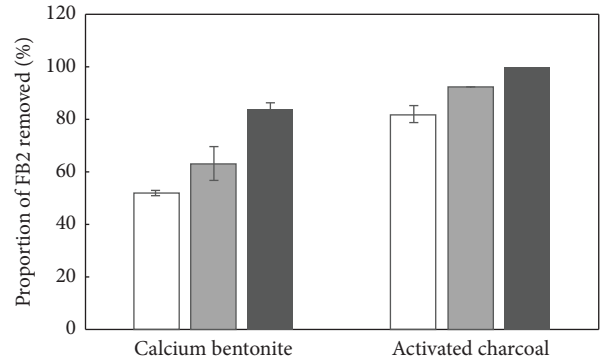


FIGURE 3: Effects of low (□) (0.25 mg/L), medium (■) (0.5 mg/L), and high (■) (1.0 mg/L) concentration of bentonite and activated charcoal on the proportion of FB2 removed from Chardonnay wine (mean ± SE, n = 3). Same letters within each treatment indicate no statistical difference as analysed using least significant difference test (LSD) (P = 0.05).

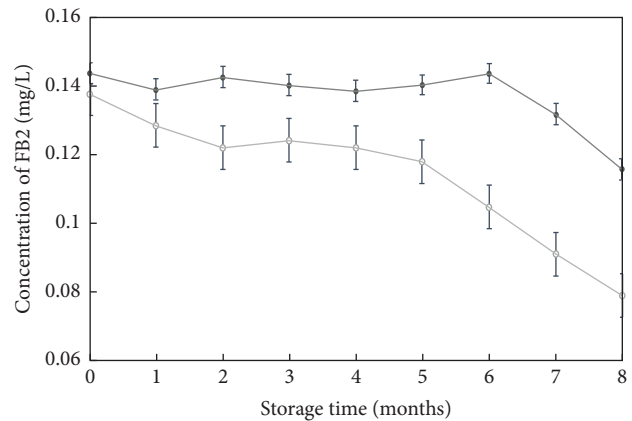


FIGURE 4: Effect of the storage of chardonnay wine at 13°C (●) and 25°C (○) on the concentration of fumonisin B2 (FB2) (mean ± SE, n = 3).

did not, however, perform mass balance in calculating the concentration of FB2, and that would also have partly contributed to the overestimation of the FB2 concentration in juice samples.

The second most important step in white-grape vinification, which contributed to the removal of the fumonisins, appeared to be solid separation (racking) after fermentation. After racking, 80% of fumonisin found in juice samples was lost. Lewis [21] reported a maximum of 77% loss of FB2 in white wine after racking during vinification of *V. rotundifolia* (cv. Carlos) grapes. Similarly, fermentation resulted in the removal of almost all FB1 from contaminated corn samples, mainly through binding by-products that were removed after fermentation [41]. Likewise, removal of solids after fermentation resulted in a reduction of the amount of OTA in red, white, and rose wines (Leong et al., 2005) [2, 20, 42], however, the proportion of OTA removed was relatively low compared to fumonisins. This difference may be attributed to the differences in the chemical structures of the two toxins, which may have some impact on binding mechanisms to solid particles such as dead yeast cells.

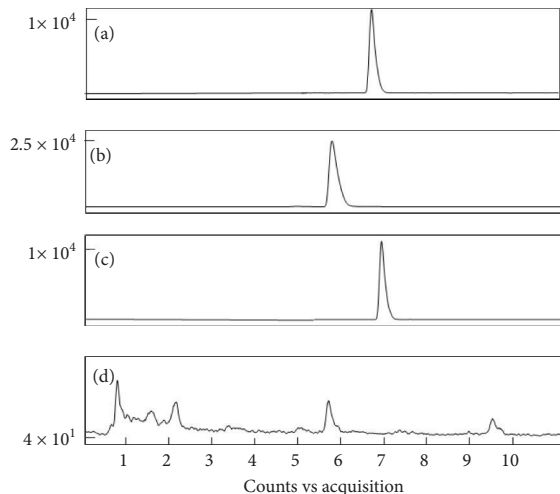


FIGURE 5: Total ion chromatograms of (a) FB2 (retention time (RT) 6.3 min) and (b) HFB2 (RT 5.6 min). Wine samples spiked with FB2 and stored for 8 months showing peaks for FB2 (c) and HFB2 (d). MRM transitions: FB2-706 > 512; 706 > 336, HFB2-390 > 372; 390 > 354.

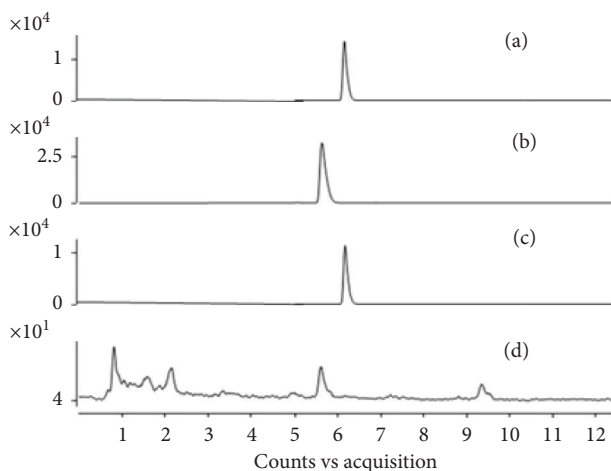


FIGURE 6: Total ion chromatograms of (a) FB2 (retention time (RT) 6.3 min) and (b) HFB2 (RT 5.6 min). Wine samples spiked with FB2 and stored for 8 months showing peaks for FB2 (c) and HFB2 (d). MRM transitions: FB2-706 > 512; 706 > 336, HFB2-390 > 372; 390 > 354.

A small amount of fumonisins (approximately $1 \mu\text{g}$) was detected from all the control wine samples. In contrast, no fumonisins were detected from juice samples from control grapes. Filtration of wine through a $0.45 \mu\text{m}$ filter led to a reduction of 80% of the amount of OTA found in wine [43]. In the current study, juice samples were filtered using $0.2 \mu\text{m}$ syringe filters prior to LC/MS/MS analysis, which would have possibly removed the minor amount of fumonisins remaining in juice. Nevertheless, during fermentation, the amount of fumonisins found in the original juice samples would have passed into wine due to the increase in ethanol concentration in the ferments. The equipment used in vinification may also be a source of contamination of wine

for example with OTA [2]. In the current study, however, all the equipment used for vinification was cleaned, and therefore, this is not a possible reason for the presence of fumonisins in control wine samples. Although the cause for the presence of a small amount of fumonisins in control wine is unknown, it does not impact the results and the conclusion of this study.

In the present study, about 34% of the fumonisins found in juice samples were not present collectively in wine and lees samples after fermentation. This represents 4.3% of the total fumonisins found in grape samples. It is likely that some fumonisin would have been lost due to the biological or chemical transformation during fermentation, as previously noted for other fungal toxins [44–47]. Another possibility is that some fumonisins may have remained with minor sedimentation, which was noted in wine samples after cold stabilisation, and that was removed during centrifugation or filtration before the LC-MS/MS analysis. Nevertheless, in the present study, most of the fumonisins found in grape samples were recovered from the marc and lees (90%), and only 3% passed into the wine.

Cold stabilisation of wine samples for 30 days at 4°C had negligible effect on reduction of fumonisins in the wine samples. This is consistent with previous studies that fumonisins are less prone to degradation under low temperature conditions during storage [21, 48].

Wine is exposed to variable temperature conditions during handling and transport in addition to wine storage. Therefore, the temperature levels of 13°C (for storage in a fridge/cellar) and 25°C (for ambient temperature), were chosen to cover such conditions that wine is exposed to, in investigating the effect of temperature on the stability of FB2 in wine. Data from the experiment indicated that FB2 was less stable at 25°C compared to 13°C in Chardonnay wine (pH 3.2); however, neither temperature was influential enough to completely eliminate FB2 over the storage period of 8 months. At 25°C , the rate of reduction of FB2 was slightly higher than at 13°C and, therefore, storage for another 4 months at 25°C may completely remove FB2 in wine samples. At 13°C , however, at least 6 months of extra storage may be required to achieve complete removal. Nevertheless, results indicated that the reduction of FB2 in wine samples may possibly be related to the hydrolysis of FB2, as a small peak-related HFB2 was detected in wine samples stored for 8 months at 25°C . The amount detected was below the LOQ and does not explain the observed loss of FB2. Further research would be required to ascertain whether HFB2 accumulates in wine over a longer storage time. An understanding of hydrolysed fumonisins in wine is important as some research conducted using mammalian cell lines has revealed that these compounds are also cytotoxic [49, 50]. In contrast, long storage of white wine may lead to alterations to the flavour and aroma profile, depending on the style of wine [51, 52]. Therefore, storage of wine samples may not be an effective method for eliminating fumonisins from wine.

As the vinification did not eliminate all fumonisins found in grapes, the removal of fumonisins in white wine was attempted using commercial fining agents that are used for clarification of white wine. Among the five

agents tested, activated charcoal was the most successful agent in the elimination of FB2 in wine. Activated charcoal also proved to be effective in the elimination of OTA and off-flavour compounds in wine [30, 32, 33, 53]. Its usage at high concentrations has, however, been reported to have a negative impact on wine quality parameters [35]. Nevertheless, in the present study, it was shown that activated charcoal at 0.25 mg/L was sufficient to remove 80% of FB2 in wine within 48 h of incubation. Other studies have shown that 80–100% removal of OTA can be achieved using activated charcoal at a concentration of 0.5–1 g/L depending on the concentration of OTA in the wine and the incubation period [33]. Therefore, the results suggest that using activated charcoal at a dosage of 1 g/L, which is within the AWRI recommendation for the clarification of white wine, may eliminate both OTA and fumonisins.

Calcium bentonite was the second most effective agent in the removal of FB2 in white wine. In the wine industry, calcium bentonite is commonly used to remove excess protein that can result in haze formation in wine [54]. The current recommended dosage for calcium bentonite for the purposes of fining wine in Australia ranges from 0.2–1 g/L. Based on the results from this study, calcium bentonite, when applied at a rate of 1 g/L, removed 80% of FB2 in a 48 h period. There are conflicting results in the literature for the use of calcium bentonite for the removal of OTA. This is presumed to be due to the presence of excess protein in wine, which may interfere with the efficiency of OTA removal [30, 32]. Where removal of OTA and FB2 in wine is required, integration of another agent such as activated charcoal may be needed. Also, calcium bentonite has been found to remove aflatoxins B1 and B2 in both red and white wine [55] effectively when used at a concentration of 1.2 g/L. Therefore, usage of calcium bentonite as a fining agent may contribute to elimination of mycotoxin contamination at least partially. Further studies, however, may be warranted to identify the dosage and the exposure time needed to remove different toxins depending on the type of wine.

Among the fining agents tested, PVPP, yeast hulls, and NO (OX) did not remove FB2 effectively at the concentration and contact time used in this study. In another study, a maximum of 30% removal of FB2 in red wine was achieved with PVPP at a concentration of 10 g/L after 2 h of contact time [29]. In the present study, usage of PVPP at a concentration of 0.8 g/L resulted in a maximum of 28% FB2 removal in white wine after 24 h of contact time. Although the proportion of FB2 removal in both the studies was similar (30 vs. 28%), the concentration of PVPP needed to remove FB2 in red wine was eight times higher than it was for white wine as observed in our study. This discrepancy could possibly be related to the affinity of PVPP towards the phenolic compounds profoundly present in red wine, which may interfere with the removal of FB2 [29, 54, 56]. Similarly, previous studies have found that the effect of PVPP on the removal of OTA in both red and white wine is minimal [30, 32]. Therefore, it can be concluded that

PVPP may not be an effective fining agent in the removal of both FB2 and OTA, at least within the dosage recommended by the AWRI. Yeast hulls appeared, however, to have variable effects in the removal of OTA depending on the concentration and the wine type (red/white) [32]. Given that in the current study, yeast hulls, when used at a concentration of 1 g/L removed 21 and 35% of FB2 after 24 and 48 h contact time, respectively, further studies may be warranted to test different concentrations and incubation times for the removal of FB2 in both red and white wine. Among the five fining agents tested, NO (OX), a new fining agent made up of polysaccharides of non-animal origin with an ability to remove excess iron and copper in wine was shown to be the least effective agent in the removal of FB2 in white wine.

Based on the vinification data obtained from this study, it could be speculated that the risk associated with exposure to fumonisin through wine consumption may be less compared to other food commodity products that are contaminated with this toxin, as vinification removed a large proportion of fumonisins found in grape samples through solids separation. Consumption of foods commonly contaminated with fumonisin toxins, however, together with wine may potentially lead to the daily tolerable intake (PMTDI) of 2 µg/kg of body mass per day being exceeded calculated based on the no observed effect level (NOEL) and a safety factor of 100 recommended by the joint FAO/WHO Expert Committee [57]. Therefore, reduction of fumonisins through other means may be useful to minimise the risk of exposure through the consumption of wine.

5. Conclusions

In summary, under the conditions used in this study, white wine vinification resulted in the removal of 90% of fumonisins found in grape samples and only 3% was transferred into wine. Similar to previous reports related to other fungal mycotoxins, the majority of fumonisins were removed with the solid materials separated during vinification. Although the amounts of fumonisins detected in the final wine samples from this study were small due to the low amounts found in infected grapes, fumonisins may potentially occur in higher quantities in wine from other vineyard locations where *Aspergillus* infection with fumonisin-producing strains are prevalent. Also, vinification of red grapes may lead to more fumonisins passing into wine due to the differences in processing between red and white grapes. Data from the stability experiment suggest that fumonisins remain in wine during storage, and thus, storing wine for periods of time cannot be regarded as an effective way to eliminate fumonisin contamination. Observations in this study on the use of fining agents suggest that activated charcoal and calcium bentonite, when used at the recommended dose removes more than 80% of FB2 in white wine. Considering the health risk associated with fumonisins, monitoring *Aspergillus* contamination in vineyard locations with high potential for toxin production may be beneficial to reduce the passage of this mycotoxin from grapes to wine, and consequently, minimise the risk to human health.

Data Availability

The data support the findings are available from the corresponding author upon reasonable request.

Conflicts of Interest

The author's declare that they have no conflicts of interest.

Authors' Contributions

All authors have significantly contributed to the manuscript and are in agreement with the content.

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Supplementary Materials

Figure S1. Schematic representations of major steps in white winemaking and of the sampling process used in this study. Control; 0% infected; low, 25% infected; medium, 50% infected; high, 100% infected. Table S1. Effect of the proportion of *Aspergillus welwitschiae* (YO 1.2) infection on the composition of wine made from *Vitis vinifera* cv. Chardonnay grapes. (*Supplementary Materials*)

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