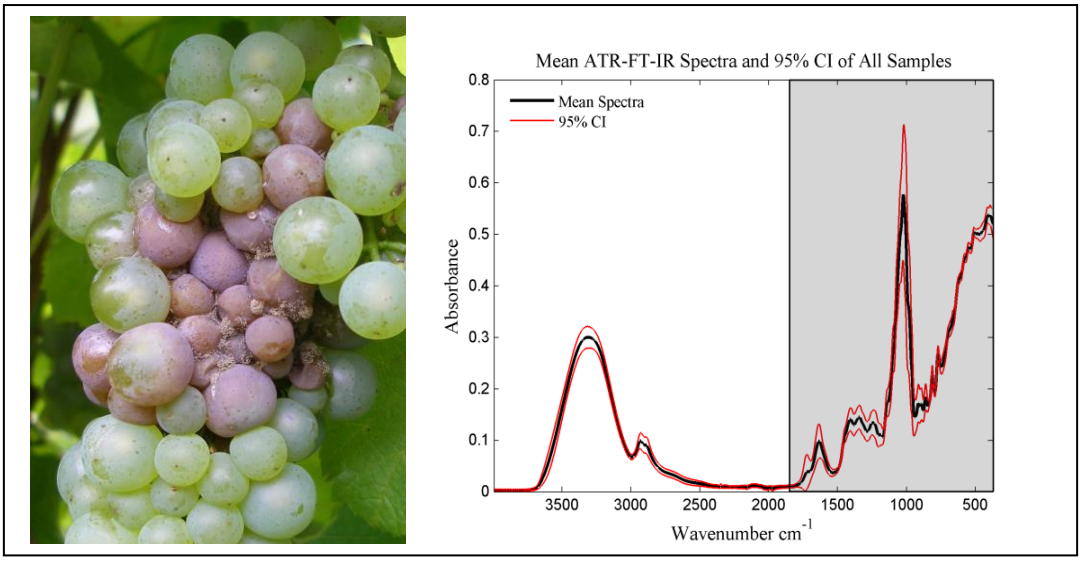
ASSESSMENT OF FUNGAL ROTS OF GRAPES AND THEIR IMPACTS ON WINE QUALITY



FINAL REPORT to

AUSTRALIAN GRAPE AND WINE AUTHORITY

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Assessment of fungal rots of grapes and their impacts on wine quality

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Abstract

This project investigated techniques to differentiate three bunch rot pathogens, *Botrytis cinerea*, *Aspergillu*s and *Penicillium*. Estimates of total fungal biomass examined chitin and ergosterol content of infected tissues. Laccase enzyme activity was variable, *B. cinerea* produced more laccase than either *Aspergillus* or *Penicillium*. Isolates of *Aspergillus* produced elevated levels of gluconic acid and had low glycerol:gluconic acid ratios. Chemical measures of off flavours and aromas using GC/MS provided an indicator of bunch rots; this technique offers an opportunity to more accurately establish bunch rot thresholds for contamination. Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FT-IR) with appropriate modelling was able to discriminate between grapes infected with *Aspergillus, Botrytis* or *Penicillium*.

1. Executive summary

Bunch rot diseases of grapes are caused by fungal moulds, typically they occur close to harvest and are found worldwide. These fungal rots result in grape yield loses and a down grading of fruit as a result of the mouldy, earthy flavours they produce which are present in wine made from bunch rot affected grapes. Disease management in vineyards is not always successful, particularly in grape growing regions that experience rainfall close to harvest. Quantification and discrimination of the type of bunch rot found in the vineyard is problematic because many of the fungi involved look similar even to the trained eye. Often more than one type of rot may be present in the one vineyard and even on the one bunch. Three such bunch rots are grey mould, caused by *Botrytis cinerea*, Aspergillus rot caused by predominantly *Aspergillus carbonarius* and *A. niger*, and Pencillium rot caused by a number of *Penicillium* species.

This project was an initial scoping study to investigate methods of bunch rot quantification and discrimination. The study focused on techniques that had the potential to be utilised in an industry setting.

Results form laccase enzyme assays as an indicator of fungal contamination of grapes were variable. Isolates of *B. cinerea* produced laccase, but the amount of enzyme produced varied depending on the culture conditions. Isolates of *A. niger* and *A. carbonarius* were found not to produce laccase under the culture conditions used. More promising results were obtained with measures of glycerol and gluconic acid ratios. Species of Aspergillus and in particular *A. carbonarius* produced higher amounts of gluconic acid than either *B. cinerea* or *Penicllium expansum* and as a consequence had a low glycerol : gluconic acid ratio.

Methodology using GC/MS analysis was developed for the quantification of a number of secondary metabolites associated with off flavours in bunch rot affected fruit and inoculated berries. All three bunch rot pathogens investigated produced a similar spectrum of secondary metabolites associated with fungal taints, including geosmin, methyl isoborneol (MIB), 1-octen-3-ol and 1-octen-3-one. Measures of these unwanted secondary metabolites may be useful in setting thresholds for bunch rot contamination in the future.

Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FT-IR) with appropriate modelling (chemometric and machine learning algorithims) was able to successfully differentiate berries inoculated with *A. carbonarius, A. niger* or *P. expansum* from the other fungi. The differentiation of *B. cinerea* from the grapes with other pathogens; also shows promise as a tool for the rapid detection of the pathogen. The ATR-FT-IR

methodology will require further validation with a greater number of isolates and samples from different cultivars.

The project examined two methods for estimating total fungal biomass in berries affected with bunch rot disease. The first method examined fungal chitin, a polysaccharide found in the cell walls of fungi. Techniques used to measure fungal chitin were found to have limited applicability for berry tissues. This was because the fungi examined degraded berry skins during the infection process, leading to interference with the fluorometeric measurements. The second method utilised a HPLC method to quantify the fungal sterol ergosterol from bunch rot affected grapes. Ergosterol was quantified from both pure fungal cultures and infected plant material and the technique may have some applicability in quantifying total fungal biomass present in grape bunches affected with fruit rotting fungi.

This pilot project has provided a base from which further research on determining thresholds for bunch rot tolerance can be set and for the further establishment of methods to objectively measure bunch rot contamination of grapes and the impact these rots have on grape and wine quality.

2. Background

Bunch rots of grapes result in a loss of both grape yield and wine quality, particularly in years that have high rainfall (e.g. Eastern states of Australia, 2009/10 and 2010 /11). A range of microorganisms are involved in bunch rots of grapes, predominantly they are filamentous fungi. It is not unusual to find a complex mixture of different fungi with different metabolic activities in the one vineyard and even on the one bunch. This makes assessing the amount of fungal rot in a parcel of fruit difficult. Many of the organisms look visually similar yet their separate impacts on wine quality are diverse.

Bunch rot of grapes is generally associated with *Botrytis cinerea* or grey mould; establishment of a regular series of international symposia on Botrytis is testament to the economic importance of this plant pathogen to grape and horticulture production worldwide (Elad, et al. 2004). Less is known about the other bunch rot diseases of grapes. Some are associated with growing grapes in subtropical regions, e.g. ripe rot and bitter rot (Steel, et al. 2007) while others are more commonly associated with diseases of the vegetative tissues, e.g. Botryosphaeria bunch rot (Wunderlich, et al. 2011). Many are regarded as opportunistic pathogens or secondary to Botrytis, yet some of their impacts on wine quality can be significant (Steel, et al. 2013). Certain isolates of some species of Aspergillus produce the mycotoxin, ochratoxin A, a human carcinogen (Leong, et al. 2007,Serra, et al. 2006,Vega, et al. 2012). Isolates of Penicillium also produce other mycotoxins such as patulin (Diaz, et al. 2011), although these tend not to survive the fermentation process. Most, if not all bunch rot fungi lead to the accumulation of unwanted flavours and aromas in grapes and wine. These compounds include 2-methylisoborneol, geosmin, 1-octene-3-ol and 1-octen-3-one. These compounds have a mushroom, mouldy or earthy odour. Little knowledge exists on thresholds for bunch rot tolerance; the information that is available largely relates to Botrytis grey mould (Ky, et al. 2012). As objective measures of bunch rot assessment are lacking, and the tools that are currently available have not been standardised, establishments of bunch rot thresholds is difficult. This issue is compounded because many of the bunch rots look visually similar when examined by the naked eye (Steel 2014), yet the individual effects of different species and potentially different isolates of the one species may vary.

Bunch rot disease control often fails especially in seasons characterised by rain close to harvest. The adaptability of fungi, and in particular *B. cinerea* means that fungicide resistance is common and spread worldwide (Korolev, et al. 2011,Latorre and Torres 2012,Leroch, et al. 2011,Rosslenbroich and Stuebler 2000). Many fungicides that have the potential to effectively control bunch rot diseases cannot be used close to harvest because of withholding periods or lack of product registration (Essling and Lord 2014). While canopy

management practices have some success in controlling some bunch rots, this can lead to a greater incidence of other bunch rots, particularly if associated with sun burn damage to fruit.

Field assessment of bunch rots generally involves a visual inspection of a representative number of bunches in the vineyard. Disease incidence and severity scales are used, but these are subjective, prone to operator error and often do not detect fungal rots that might be deep within the interior of a tightly packed cluster. Furthermore differentiation of the different bunch rot types is difficult given the visual similarity of some of the bunch rots. Wineries concerned with the impact of bunch rots on wine quality may test fruit / must for the oxidative enzyme laccase, however, not all fungal isolates produce laccase. Commercially available kits for botrytis antigen detection are available. These provide a more accurate estimation of Botrytis than assays for laccase (Dewey, et al. 2008). Similar kits are currently not available for the other fungi involved in bunch rots. Estimates of glucose: gluconic acid ratios are used in France to differentiating grey mould from noble rot (Ribéreau-Gayon, et al. 2006), the applicability of these methods to provide an objective measure of unwanted bunch rot contamination is not known.

Spectroscopic measurements (NIR) have been investigated for determination of powdery mildew infection of grapes with some success (GWRDC-Report-UA05/08). Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FT-IR) is a technique that involves the collection of infrared spectra of samples using reflectance spectroscopy and is a simple and rapid procedure that has been used to measure non structural carbohydrates in grapevine tissue (Schmidtke, et al. 2012), some inorganic nutrients in grapevine petiole tissues (Smith, et al. 2014) and may have some applicability to discriminate bunch rot type.

This project was a preliminary scoping study and aimed to compare methods of bunch rot assessment using detached grape berries artificially inoculated in the laboratory. The techniques investigated included laccase enzyme assays, measurements of glycerol and gluconic acid ratios, GC-MS methods for the detection of off flavours associated with earthy and mouldy aromas and Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FT-IR). A further aim was to investigate methods to measure total fungal biomass in grapevine tissues. Our observations have concentrated on three bunch rot diseases, *B. cinerea* (grey mould), *Aspergillus* and *Penicillium* (Figure 1) with the intention to extend these studies to include naturally infected bunches with mixed fungal populations from vineyard field sites. The project was integrated with a series of wine industry workshops and seminars held at various locations in Australia at various stages during the lifetime of the project.

(a)



(b)



(c)

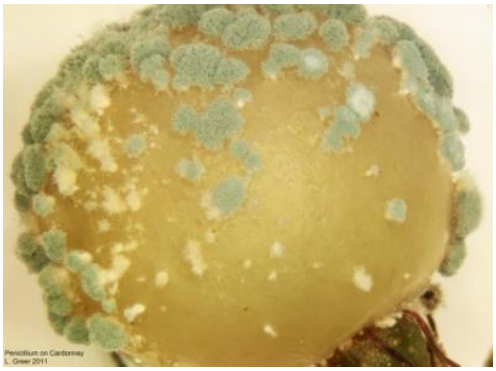


Figure 1: Bunch rot diseases of grapes investigated in this project. (a) Botrytis cinerea *(b)* Aspergillus *and (c)* Penicillium expansum*.*

3. Project aims and performance targets

The following table summarises the planned outputs and performance targets as proposed in the original funding application for this project. Achievements in relation to these outputs is discussed in subsequent sections of this report.

Outputs and target dates 2012–13

Year 1	Output	Target Date
	Staff appointed	14/01/13
	Establish standard assay procedures for laccase and immuno detection of Botrytis antigens. Establish ATR-FT-IR methodology	28/02/13
	Infection studies using grape bunches under controlled environment conditions completed	31/05/13
	Established correlations between berry measurements using ATR-FT-IR compared with other techniques of bunch rot assessment.	28/06/2013

Outputs and target dates 2013–14

Year 2	Output	Target Date
	Preliminary knowledge of potential new objective measures of bunch rots disseminated to industry	31/07/2013
	Knowledge of new objective measures of bunch rots disseminated to industry	20/12/2013
	Methodology evaluated for mixed bunch rot samples grown under field conditions.	21/03/2014
	Information on objective measures of bunch rot assessment available to the wine industry through at least two extension mechanisms.	30/04/2014

4. Methods

Fungal isolates

Fungal isolates used in this work were obtained from either the existing NWGIC culture collection maintained at the Wagga Wagga campus of Charles Sturt University, or purchased from CSIRO (CSIRO Animal, Food and Health Sciences, North Ryde, NSW). Isolate details are presented below in Table 1.

Table 1 Details of fungal isolates and their origins

Fungal species	Isolate number	Original host and location	Culture Collection Source
Aspergillus carbonarius	FFR 5690	*V. vinifera* cv Semillon, Griffith, NSW.	CSIRO
Aspergillus niger	FFR 5575	*V.vinifera* cv Sultana, Merbein, VIC.	CSIRO
Botrytis cinerea	Bc 1	*V. vinifera*, Hunter Valley, NSW	NWGIC
Botrytis cinerea	Bc 2	*V. vinifera*, Mudgee, NSW	NWGIC
Botrytis cinerea	Bc 3	*V.vinifera*, Tumbarrumba, NSW	NWGIC
Penicilllium expansum	PEN 1	*V.vinifera,* unknown	NWGIC

Berry inoculation

Fungal spore suspensions were prepared for berry inoculation by growing isolates on plates of potato dextrose agar (PDA) incubated at 27 ºC, under a 12hr light/dark photoperiod until spores developed. Spores were washed from the plate with 4 mL sterile distilled water and spores counted using a haemocytometer. Spore concentrations were adjusted to 10^6 spores per mL using sterile distilled water. Disease-free *Vitis vinifera* (cv Chardonnay) grape berries were collected from either the Charles Sturt University vineyard at Wagga Wagga or vineyards in Tumbarumba or Griffith. During periods of the project when field-grown wine grapes were not available some experiments were conducted on table grapes (cvs Flame Seedless, Red Globe and Thompson Seedless) purchased from a local supermarket.

Individual berries were removed from bunches by cutting the pedicel to ensure that the brush remained intact, and surface sterilised using sodium hypochlorite (1% v/v, plus 0.05% v/v Tween 20®, 2 mins) followed by three successive washings in sterile distilled water. Berries

were placed into the wells of a microtitre plate (24 well, flat-bottom, Iwaki Microplates) and inoculated by placing a 10 µL drop of a spore suspension (10^6 spores/mL in sterile distilled water) onto the apex of the berry surface without wounding the berry. Control berries were inoculated with 10 µL of sterile distilled water. Each replicate consisted of 24 individual berries and there were three replicates per treatment. Twenty millilitres of sterile water were added to the base of each plate, surrounding the wells, giving a 100% relative humidity. Lids were placed on the microtitre plates, stacked up to six high, sealed with cling film and incubated at 25 ºC. Each replicate consisted of one microtitre plate containing 24 berries. Replicates were frozen at -20 ºC for further analysis at various time points post inoculation (Figure 2).

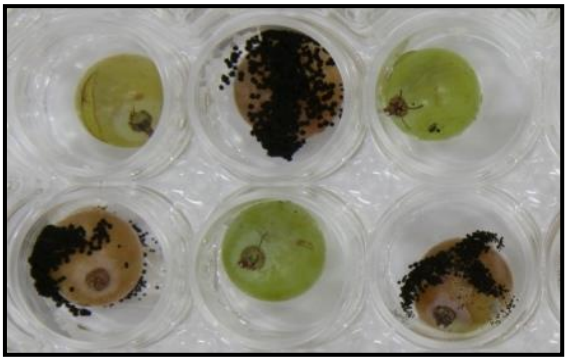


Figure 2: Section of a 24-well Iwaki plate containing chardonnay berries inoculated with Aspergillus carbonarius *(FFR 5690). Plate was incubated at 25 ºC for five days under a light (12hr) / dark (12hr) cycle. Berries represent different levels of infection.*

Bunch inoculation

Apparently healthy / uninfected *Vitis vinifera* (cv Chardonnay) bunches (average Baume 13.9) were collected from commercial vineyards in the Riverina. Any berries with visible signs of damage such as sun burn were removed from bunches prior to use. Whole bunches were dipped in a plastic tub (25 L volume) containing sodium hypochlorite (0.4% v/v) for 2 minutes. Bunches were then rinsed three times with distilled water prior to use. Bunches were wounded by tapping the surface of two sides of the bunch with a sterile wire brush. Whole bunches were then dipped in a beaker containing 500 mL of the relevant fungal spore suspension. Fungal spore suspensions were prepared as described above for berry inoculation except final spore concentrations were adjusted to 10^4 spores / mL. Control un-inoculated bunches were dipped in sterile distilled water. Excess moisture was allowed to drain from the bunch prior to placing the bunch in a ziplock plastic bag and recording the weight. Control (Day 0) bunches were frozen immediately; all other bunches were incubated in the dark at 25 C. After five days of incubation, bunches were then crushed and homogenised and the total volume recorded. Juiced bunches were divided into six aliquots of known volumes for subsequent analyses and stored at -20 ºC.

Laccase enzyme assays

Initial screening of fungal isolates took place by growing isolates on potato dextrose agar (PDA) plates supplemented with 2,2'-Azino-bis 3-ethylbenzthiazoline-6-sulfonic acid (ABTS) (0.01% w/v) as a substrate for laccase (Liao, et al. 2012).

Quantitative determination of laccase was carried out using ABTS as a substrate and the change in absorbance at 436nm monitored using a spectrophotometer (Guetsky, et al. 2005). Laccase was assayed from culture filtrates of the isolates grown in a laccase inducing medium (LIM) (as described by (Guetsky, et al. 2005), and from sets of individual grape berries (10) inoculated with the fungi. There were three replicates per treatment. Berry homogenates or culture filtrates were centrifuged (10 mins, 3000g) to remove suspended material. PVPP was then added to the supernatants which were then filtered (millipore 0.45 µm) and the filtrate collected. The filtrate was further concentrated using a 30,000kDa molecular weight filter (Centricon 30, Amicon). The filtrate was then assayed for laccase activity.

For the ABTS laccase assay the following were added to a 1 mL cuvette in consecutive order, 0.1 M phosphate buffer pH 6.0, (0.8mL); ABTS (26mg/mL), (0.1 mL); and either culture filtrate or berry homogenate (0.1mL). The methodology was initially trialled with a standard laccase enzyme preparation from *Pleurotus ostreatus* (Sigma-Aldrich, Sydney). Absorbance at 436 nm was measured for up to 1 hour at 22 ºC (Guetsky, et al. 2005). Total protein content was measured using the Bradford method (Bradford 1976) and enzyme activity expressed in µg ABTS converted / min / mg total protein.

Glycerol: Gluconic acid ratio

The ratio of glycerol to gluconic acid can be used to discriminate between grey mould and noble rot of grapes (Ribéreau-Gayon, et al. 2006). Fruit affected with the less desirable grey mould has elevated levels of gluconic acid. The aim of this aspect of the work was to determine if differences existed in the glycerol : gluconic acid ratio in fruit affected with different fungi.

Berry homogenates were thawed and 1mL samples were analysed for glycerol and gluconic acid enzymatically with Megazyme Glycerol and D-Gluconic Acid kits (Megazyme International Ireland Ltd.) using a Konelab Arena 20XT auto analyser. Glycerol calibration was achieved with calibration values of 0, 1.32, 2.2, 3.3, 4.4 and 6.6 g/L. For the gluconic acid assay, the calibration was established by measuring 0, 0.125, 0.250, 0.417, 0.625, 0.83

and 1.25g/L gluconic acid calibration values. Tests were verified using a combined standard that contained 0.25g/L gluconic acid and 0.4g/L glycerol.

Analysis of off flavours by GC/MS

The production of off flavours in grapes and wine as a result of prior fungal bunch rot infection in the vineyard is a known phenomenon (La Guerche, et al. 2006). However, the chemical nature of these off flavours has not been fully characterised. Furthermore thresholds for bunch rot contamination and fruit rejection are poorly understood (Steel, et al. 2013). In this aspect of the project the formation of off flavours in grapes infected in the laboratory with *Aspergillus, Botrytis* or *Pencillium* species was investigated.

Berries juices were accurately diluted 1:5 in ultrapure water, followed by pH adjustment to 7 by adding 1 M NaOH. Samples (10mL) were accurately pipetted into 20mL headspace vials containing 3.0g NaCl and 10 µL of internal standards. The concentrations of internal standards in each sample were: 2-methyl-d3-isoborneol 100ng/L, methyl-isobutyl-ketone 97ng/L, 2-octanol 60.2ng/L and D3-geosmin 30ng/L. With the exception of 2-methyl-d3-isoborneol which was obtained from CDN isotope (Quebec, Canada), all other standards were obtained from Sigma-Aldrich, Sydney. The vials were immediately capped with magnetic screw caps with Teflon liners. Sample vials were stored at 8°C until analysis, transported to a heater block set to 65˚C and equilibrated with agitation for 2 minutes. A DVB/CAR/PDMS SPME fibre was inserted into the headspace of the vial for 44 minutes, with vial agitation, and then inserted into the injector block at 260°C in splitless mode for 2 minutes. An Agilent 7890A GC fitted with a fused silica capillary column (DB-Waxetr, 60m x 0.25 mm id 0.25µm film thickness) interfaced with an Agilent 5975C mass detector was used for separation of compounds and quantification. The GC oven temperature program commenced at 40°C for 3 minutes; raised to 115°C at a rate of 5°C/min; raised to 166°C at a rate of 3°C/min and a final increase to 210°C at a rate of 10°C/min and held for 12 min. The total run time was 51.4 minutes. The flow rate of ultra high purity helium gas was constant at 3mL/min. The MS source, quadrapole and transfer line temperatures were set to 230°C, 150°C and 275°C respectively and compounds detected using selective ion monitoring (SIM) at -70 ev (details in appendix). Data were analyzed using a one-way ANOVA followed by Fisher's tests from Minitab (Minitab Inc., State College, PA) version 17. Significance of differences was defined at $P < 0.05$.

Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FT-IR)

An Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FT-IR) is a technique that has been applied to the analysis of a number of biological systems (Kazarian and Chan 2013). ATR-FT-IR was investigated for the assessment of fungal rots caused by *Aspergillus, Botrytis* and *Penicillium.* Berry / bunch homogenates were thawed and 10 µL aliquots analysed using an Alpha –P Bruker ATR FT IR Spectophotometer (Bruker Optics). After some initial methodology development, samples were routinely heated to 60 ºC for 5 mins prior to analysis.

Determination of fungal biomass in infected berries

Two methods of estimating fungal biomass in infected plant tissues were investigated in this project; measurements of fungal chitin (Ayliffe, et al. 2013) and measurements of the fungal sterol, ergosterol (Porep, et al. 2014).

Fungal chitin as a measure of fungal biomass in plant tissues - A method for estimating fungal biomass based on the chitin content of wheat and grapevine leaves infected with rust and powdery mildew respectively has recently been developed (Ayliffe, et al. 2013). The method is based upon chitin detection using wheat germ agglutinin (WGA) conjugated to a fluorophore, fluorescein isothiocynatae (FTIC) which absorbs at 485 nm and has an emission wavelength of 535 nm. The suitability of this method for estimating fungal biomass in bunch rot affected berry tissue was investigated using the published method. In brief, the method was as follows. Pre-weighed grape berries previously inoculated with one of the fungal bunch rot pathogens used in this project were placed in KOH (1M), autoclaved and then successively washed gently with Tris buffer (50mM, pH 7.0). Un-inoculated berries served as controls. Berries were then homogenised and a known volume (40 – 200 µL) of the homogenate was taken and 10 µL of WGA-FITC (Sigma-Aldrich, Sydney) (1 mg/mL solution) added. The mixture was left to stand for 10 mins at room temperature and then centrifuged (1000g, 3 mins) and the pellet re-suspended in 100 µL of Tris buffer (50mM, pH 7.0). The samples were then transferred to a microtitre tray for fluorometry. Fluorometric measurements were conducted with a Cary Eclipse Fluorometer with 485 adsorption and 535 nm emission wavelength. Standard curves were prepared using chitin from shrimp shells (Sigma- Aldrich, Sydney).

Ergosterol as a measure of fungal biomass in plant tissues - The principal sterol found in fungal cell membranes is ergosterol (Mercer 1984) while grapevines along with other plant species contain a range of other sterols, most notably sitosterol and stigmasterol (Le Fur, et

al. 1994). The suitability of a recently published method (Porep, et al. 2014) for estimating fungal biomass from vine tissues was investigated.

Fungal mycelium (*B. cinerea*) cultured in potato dextrose broth (PDB) was harvested by filtration and the wet weight determined. The mycelium (approx. 100 mg) was then transferred to a 250 mL round-bottom flask and 50 mL of ultrapure water added. Then 75 mL methanol, 50 mL ethanol and 10 g potassium hydroxide (KOH) were added to the flask. The flask was assembled to a condenser unit and saponified with refluxing for 30 minutes. The content of the flask was allowed to cool down and filtered through a fluted filter paper (Whatman No.1) into a 250 mL separating funnel, rinsing the flask once with 25 mL ultrapure water. Ergosterol was twice extracted from the alcoholic phase with 50 mL n-hexane. The upper organic layers from the two extractions were combined and filtered over anhydrous sodium sulphate, rinsing once with 5 mL n-hexane. The hexane in the extract was removed by rotary evaporation to about 5 mL of extract. This volume of extract was quantitatively transferred into a 25 mL scintillation vial and dried under a continuous stream of nitrogen gas. The dried extract was kept at -20 ^{o}C pending HPLC analysis. A similar procedure was carried out using homogenates from whole bunches of grapes inoculated in the laboratory with each of the fungal species used in this project. For the grape samples, 20 g of homogenates were used for the extraction.

For HPLC analysis, the dried extract was dissolved in 5 mL methanol assisted by sonication. The solution was filtered through a solvent compatible filter into an HPLC vial. HPLC analysis was performed on a Waters 600E Multisolvent Delivery System equipped with a Waters 2996 PDA detector, a Waters 717 plus Autosampler and a Waters temperature control system (Waters, Milford, USA). Sterols were separated using a Varian SP–C18 – 5, 4.5 µm, 4.0 mm X 15 cm column kept at 25 ^{o}C. The mobile phase consisted of methanol/water, 95:5 (v/v). The Autosampler was programmed to inject 25 µL of extract and elution was done isocratically, at a flow rate of 0.8 mL/min with detection at 282nm. The total run time was 40 minutes. External calibration was prepared using a pure ergosterol standard (Sigma-Aldrich, Sydney, Australia). A stock solution of 400 mg/L ergosterol in methanol was prepared and serially diluted into a series of working standard solutions ranging from 0 to 400 mg/L.

5. Results and Discussion

Laccase

Initial screening of fungal isolates for laccase production involved culturing fungi on PDA plates supplemented with ABTS which serves as a substrate. Upon oxidation ABTS forms a green colour which can be detected. Thus actively growing fungal cultures that produce laccase turn the surrounding medium green which can be readily detected with the naked eye. Based on this qualitative estimation, the isolate of *B. cinerea* used for this study produced laccase while the species of *Penicilium* and *Aspergillus* either did not produce laccase or produced the enzyme at levels below the limit of detection (Figure 3).

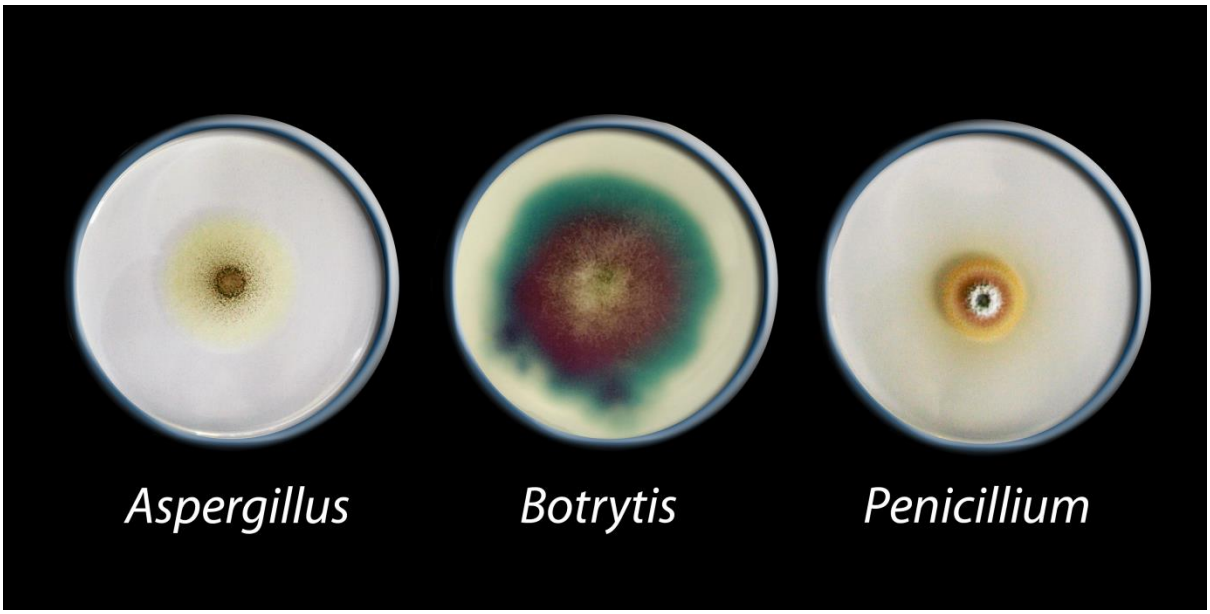


Figure 3: Growth of Aspergillus carbonarius, Botrytis cinerea *(Bc1) and* Penicillium expansum *on PDA supplemented with the laccase substrate ABTS. Green colouration indicates presence of laccase enzyme activity*

These qualitative results were largely confirmed using homogenates from berries inoculated with fungal isolates and culture filtrates of the fungi grown in laccase inducing medium (LIM). Laccase was assayed using ABTS as a substrate and the change in absorbance at 436 nm monitored. Neither *A. niger* or *A. carbonarius* were found to produce laccase when grown in culture or when inoculated on to grape berries. Relatively low levels of laccase were detected in *P. expansum* while greater levels were found in the three *B. cinerea* isolates examined. However laccase activity varied between isolates and between experiments (Table 2) and appear to depend on the substrate used to culture the fungus. Further

methodology development is required before any conclusions can be made regarding laccase production as a means of screening bunches for bunch rot contamination, particularly in light of the fact that ABTS can also be a substrate for peroxidises and tyrosinases (Mayer and Staples 2002). Without further work it is difficult to comment on the usefulness of laccase as an indicator of bunch rot contamination or bunch rot type.

Table 2 : Laccase activity of B. cinerea, Penicillium expansum, Aspergillus carbonarius and Aspergillus niger *isolates in culture medium or when cultured on grape berries*

Growth substrate / medium	Laccase activity (µg ABTS converted / min/mg protein) Isolate*					
	Bc 1	Bc 2	Bc 3	Pe	Ac	An
Chardonnay berries	ND	ND	0.21	0.06	0	0
Red Globe berries	0.09	0.40	0.48	0.08	0	0
MS / LIM culture medium	0.99	0	0.27	0	0	0

* Bc = *B. cinerea* isolates 1,2 & 3, Pe = *Penicillium expansum*, Ac = *Aspergillus carbonarius*, An = *Aspergillus niger*

Glycerol: Gluconic acid ratios in individually inoculated grape berries (cvs Chardonnay and Flame seedless)

Studies on the glycerol and gluconic acid content of detached individual grape berries inoculated in the laboratory were initially conducted as a pilot study using a table grape variety (cv Flame seedless), purchased from a local supermarket outlet. Infected berries all had greater levels of glycerol and gluconic acid than healthy berries control. Elevated levels of gluconic acid were particularly obvious in those berries inoculated with either of the two species of *Aspergillus* examined which translated to a low glycerol : gluconic acid ratio. (Table 3)

Table 3 : Glycerol : Gluconic acid ratios in individual grape berries, cv Flame Seedless

Treatment	Glycerol g/l	Gluconic acid g/l	Glycerol : Gluconic acid
Non-inoculated	0	0.94	0
B. cinerea	3.53	2.52	1.40
P. expansum	0.87	3.14	0.28
A. carbonarius	1.07	11.80	0.09
A. niger	2.34	21.1	0.11

Following on from this initial experiment, a similar experiment was conducted with detached Chardonnay berries using a larger sample size of 30 berries per treatment. The results were consistent with the findings obtained with the Flame seedless berries. Inoculation of Chardonnay berries with each of the four fungal pathogens resulted in elevated levels of both glycerol and gluconic acid, the amount of gluconic acid in berries inoculated with *A. carbonarius* was particularly enhanced compared to berries inoculated with the other fungi (Table 4).

Table 4 : Glycerol : Gluconic acid ratios in individual grape berries, cv Chardonnay 2013. Results are the mean of 30 berries. Data was analysed using a two-way ANOVA, letters within columns indicate significant differences P <0.001.

Treatment	Glycerol g/l	Gluconic acid g/l	Glycerol : Gluconic acid
Non-inoculated (day 5)	0.39^{a}	0.25^{a}	1.59^{b}
B. cinerea	2.29^{d}	0.53^{a}	4.40^{c}
P. expansum	5.46^{e}	1.62^{b}	4.07^{c}
A. carbonarius	0.91^{b}	16.29^{c}	0.07^{a}
A. niger	1.68^{c}	1.35^{b}	1.47^{b}

Glycerol: Gluconic acid ratios in whole bunches (cv Chardonnay)

Studies on the glycerol and gluconic acid content of berries were extended to include whole Chardonnay bunches collected from the field. The non-inoculated bunches at the start of the experiment (i.e. day 0) had low levels of glycerol (0.018 g/L) and gluconic acid (0.028 g/L) which increased with incubation at 25 ºC. This observation may indicate that low levels of fungal contamination were present, possibly as latent infections in the interior of the berries which would not have been affected by surface sterilisation. This was confirmed by visual inspection as fungal growth was apparent on bunches after five days of incubation. Moist incubation in sealed plastic bags would have compounded this issue. Future investigations are likely to employ whole bunches that are incubated in paper bags which will result in a more natural simulation of field conditions. Despite the limitations of the methodology used, elevated levels of gluconic acid were found in bunches inoculated with both species of Aspergillus examined, and in particular in those berries inoculated with *A. carbonarius* (Table 5).

Table 5 : Glycerol : Gluconic acid ratios in inoculated whole bunches, Chardonnay grapes Results are the mean of 25 bunches

Treatment	Glycerol g/l	Gluconic acid g/l	Glycerol : Gluconic acid
Non-inoculated (day 0)	0.018	0.028	0.63
Non-inoculated (day 5)	0.314	0.275	1.42
B. cinerea	1.094	0.321	3.89
P. expansum	1.879	0.741	2.80
A. carbonarius	0.816	5.820	0.19
A. niger	0.940	2.761	0.37

Analysis of off flavours by GC/MS

Geosmin, methyl isoborneol (MIB), 1-octen-3-ol and 1-octen-3-one, four compounds associated with off flavours and aromas in grapes affected with bunch rot pathogens, were either not detected or found at very low concentrations in non-inoculated berries.

The concentration of geosmin and 1-octen-3-ol were significantly higher ($P<0.05$) in grapes inoculated with *A. carbonarius* and *Penicillium expansum* than in grapes inoculated with *A. niger* or *B. cinerea* five days post-inoculation (Table 6). The concentration of MIB and 1-octen-3-one were significantly higher ($P<0.05$) in grapes inoculated with *A. carbonarius* than in grapes inoculated with the other fungi investigated. Based on the limited number of isolates investigated, these results suggest that *A. carbonarius* has a greater impact on off-flavours than *A.niger, B. cinerea* and *P. expansum.*

Table 6: Levels of 1-octen-3-one, 1-octen-3-ol, methyl isoborneol (MIB) and geosmin (ng / L) in grapes affected with various fungal bunch rot pathogens five days post-inoculation. Mean values within each column followed by different letters are significantly different at $P < 0.05$.

	1-Octen-3-one	1-Octen-3-ol	MIB	Geosmin
Control (Day 0)	0.0 ± 0.0^{d}	0.0 ± 0.0^{c}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}
Control (Day 5th)	16.7 ± 9.4^{c}	77.4 ± 28.1^{b}	3.4 ± 6.7^{b}	1.4 ± 1.8^{ab}
A. niger	19.9 ± 1.7^{bc}	24.8 ± 5.2^{bc}	0.0 ± 0.0^{b}	1.8 ± 1.7^{ab}
A. carbonarius	29.6 ± 11.2^{a}	206 ± 63.1^{a}	24.2 ± 5.6^{a}	4.5 ± 1.1^{a}
B. cinerea	20.7 ± 5.6^{bc}	34.5 ± 8.7^{b}	3.3 ± 0.5^{b}	4.9 ± 0.4^{a}
P. expansum	27 ± 5.4^{ab}	204 ± 65.7^{a}	0.0 ± 0.0^{b}	5.0 ± 1.6^{a}

These studies will need to be extended to include sensory analysis of wine made from grapes infected with the different bunch rot pathogens.

Determination of fungal biomass in infected berries

Initial experiments investigated chitin as an indicator of fungal biomass in grapevine berries. Standard curves using shrimp shell chitin were prepared using the WGA-FTR method (data not shown), and preliminary experiments were conducted on Chardonnay leaves inoculated with *B. cinerea* (Figure 4).

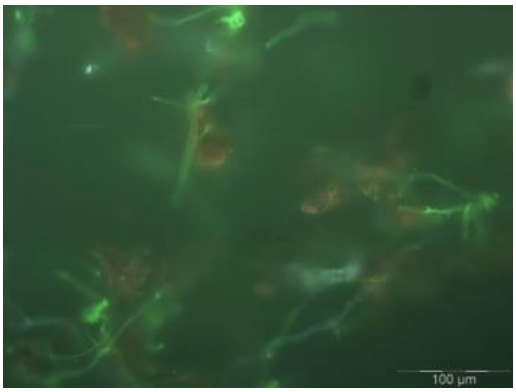


Figure 4: Vitis vinifera (cv Chardonnay) leaf inoculated with Botrytis cinerea *(Bc1) and stained using the WGA-FTR method (Ayliffe, et al. 2013). Chitin in the fungal mycelium fluoresces green.*

However the technique was found not to be particularly suitable for chitin estimations from berry tissue. The method involved staining berry skins preparations, and in those berries infected with fungi, and in particular berries infected with either *Aspergillus* or *Penicillium*, the skins become degraded as result of fungal growth, leading to inaccurate results (data not shown). For this reason further work using chitin as an estimation of fungal biomass in grape berries was not pursued.

Subsequent work investigated the fungal sterol, ergosterol as an indicator of fungal biomass in infected grapevine berries. Initially a mixture of sterol standards comprising ergosterol, cholesterol, sitosterol and stigamsterol were analysed. The HPLC method used was able to separate ergosterol from other major sterols including cholesterol, sitosterol and stigamsterol. In contrast to the other sterols, ergosterol has an absorption maximum at 282 nm which can be utilised to specifically detect ergosterol from plant material infected with fungal pathogens. Ergosterol had a retention time of 26 minutes while the phytosterols, sitosterol and stigmasterol both eluted within 5 minutes of the chromatographic analysis. Figure 5 shows a typical chromatogram of ergosterol extracted from *B. cinerea* mycelium grown in potato dextrose broth. A preliminary calculation indicates that *B. cinerea* contains approximately 16 mg of ergosterol per gram fresh weight of fungus. We recognise that different fungal species will vary in their ergosterol content and measures involving dry weight are more accurate than wet weights, however the experiment demonstrated that

measures of ergosterol have the potential to quantify fungal biomass in infected plant tissues.

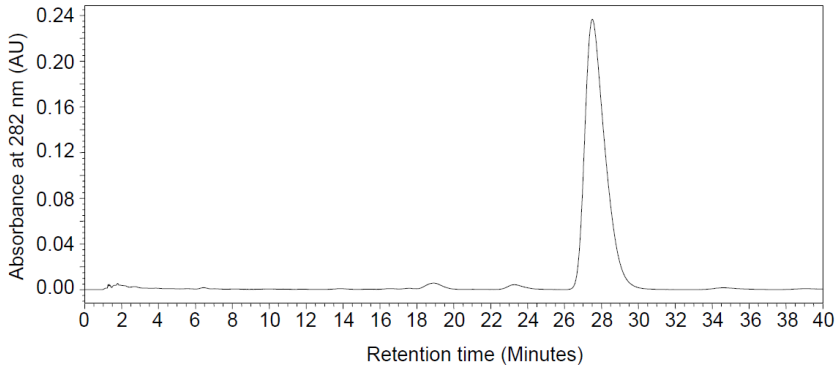


Figure 5: Typical chromatogram of ergosterol extracted from Botrytis cinerea *(Bc1) mycelium grown in potato dextrose broth for five days at 25 ºC. The largest peak is due to ergosterol.*

Representative bunches of Chardonnay grapes sourced from a vineyard in the Riverina with bunch rot disease were collected and sorted into apparently healthy and bunch rot affected fruit. Visual inspection suggested that diseased fruit was predominantly affected with grey mould although Aspergillus was present on a few berries in some bunches. Ergosterol was detected in both apparently healthy and bunch rot affected grapes, however the levels in the infected grapes were higher. Assuming *B. cinerea* contains 16 mg ergosterol per gram fresh weight of fungus (discussed above) the amount of fungal biomass present in apparently healthy and bunch rot-affected bunches was approximately 0.34g/kg and 3.34g/kg respectively (Table 7).

Table 7 : Ergosterol content of field samples of bunch rot affected grapes and an extract of B. cinerea *grown in potato dextrose broth*

Sample	Fungal biomass (g/kg)
Apparently healthy bunches	0.34
Bunches affected with grey mould	3.34

The low level of ergosterol present in the apparently healthy grapes may have been due to latent infection or wild yeasts growing on the surface of the fruit.

Further quantitative development of this methodology is still required, typical chromatograms from apparently healthy and bunch rot affected bunches are presented below in Figure 6.

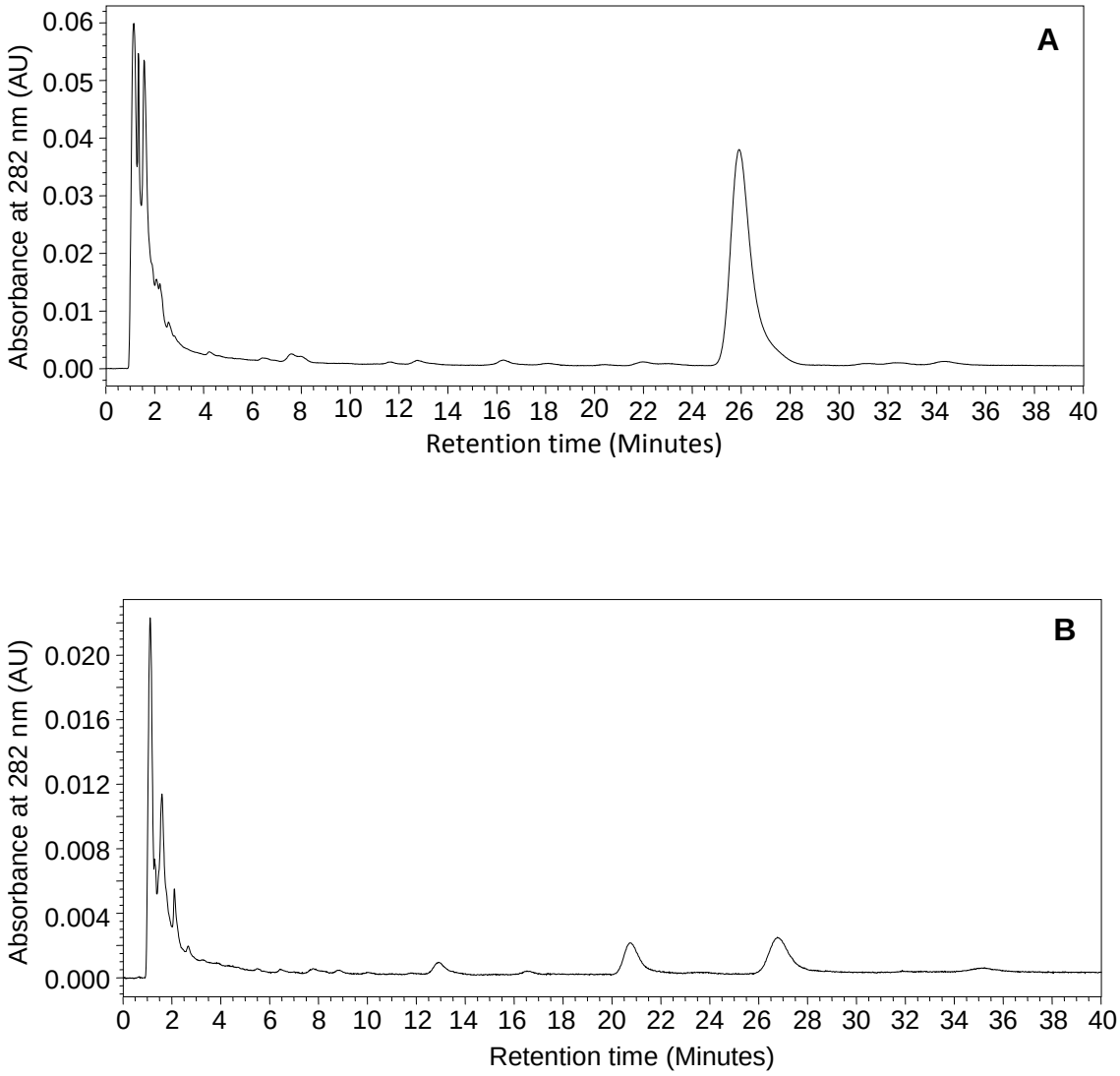


Figure 6: Typical chromatogram of ergosterol extracted from bunch rot-affected (A) and apparently healthy (B) grape bunches (cv Chardonnay). The peak eluting at 26-27 minutes is due to ergosterol.

Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FT-IR)

Establishing a method for improved sensitivity for MIR scans

Preliminary investigations using ATR-FT-IR scans of grape berry extracts were conducted to determine the potential for this technique to be utilised for pathogen detection. Berry extract samples were placed onto the ATR and scans were immediately scanned using the wavelength range from 375-4000 cm^{-1}. Samples were also scanned with the ATR temperature set at 60°C and these are presented below in Figure 7.

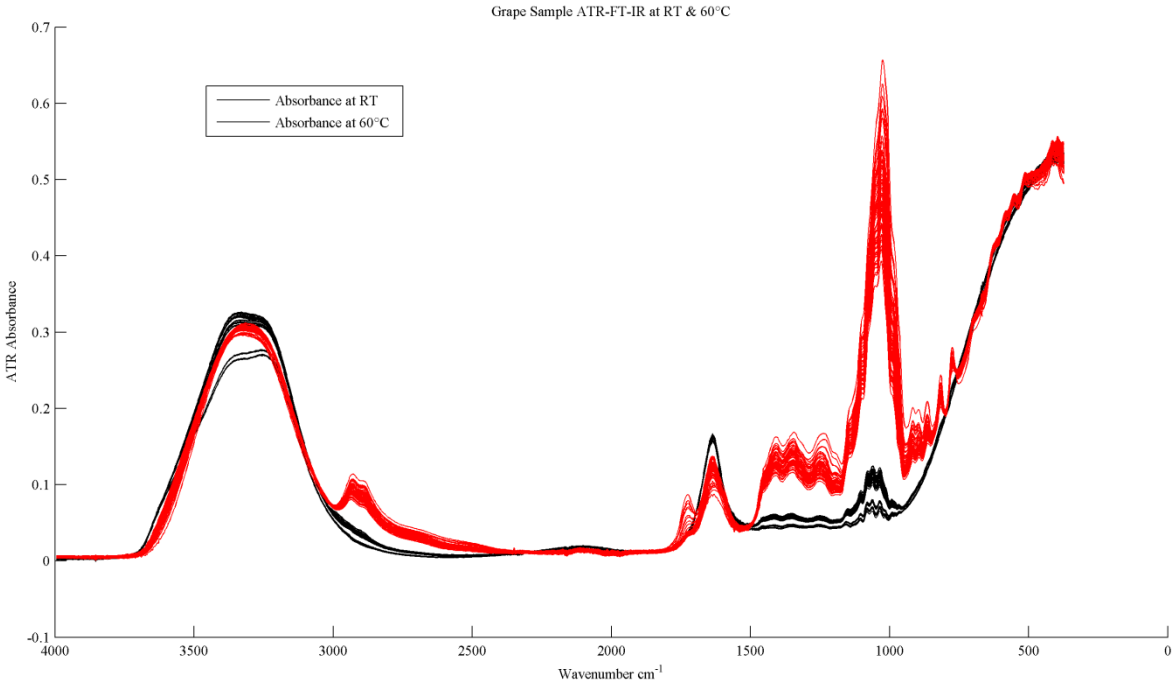


Figure 7: ATR-FT-IR scans of berry extracts conducted at room temperature and 60°C.

Inspection of the region from 375-2000 cm^{-1} indicates poor sensitivity for signal detection within this region. This region of the spectra is important as C-C; C-O and C-N bond stretching, twisting and oscillation frequencies lead to important energy absorption patterns which manifest in absorption spectra that correlate well with compositional data. To improve the sensitivity of the ATR-FT-IR data collection a series of experiments using small volumes (10µL) of berry extract with drying times ranging from 0-20 minutes at two different temperatures (40 and 60 °C) were undertaken. IR scan results are illustrated below (Figure 8) and from this small experiment it was considered that a 10µL sample placed onto the ATR at 60°C for 5 minutes was sufficient to increase the scan sensitivity.

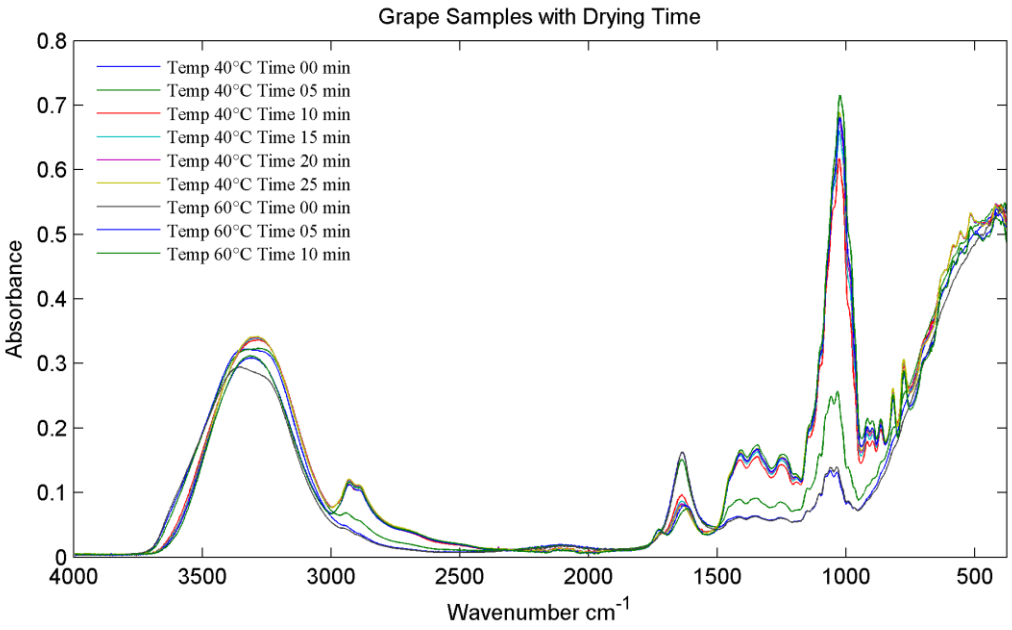


Figure 8: ATR-FT-IR scans of berry extract samples at varying time and temperature combinations.

Modelling Berry Infections by Different Pathogens -ATR-FT-IR spectra collection

Infrared spectra of grape berry juice were collected using an Alpha ATR-FT-IR spectrophotometer running OPUS 7.0 software (Bruker, Ettlingen, Germany). Prior to spectral analysis, juice samples were allowed to concentrate onto the surface of the diamond internal reflection element for 5 minutes at 60°C. Each sample was measured once with ATR spectra averaged from 64 scans over the region 375-4000 cm^{-1}. A background scan against air was conducted approximately every 10 samples. Data was converted to ascii format at a resolution of 1.4 cm^{-1} and imported into Matlab version R2012a (The Mathworks, Natick).

The mean spectra and 95% CI are illustrated below in Figure 9; the shaded area indicates the region selected for further analysis.

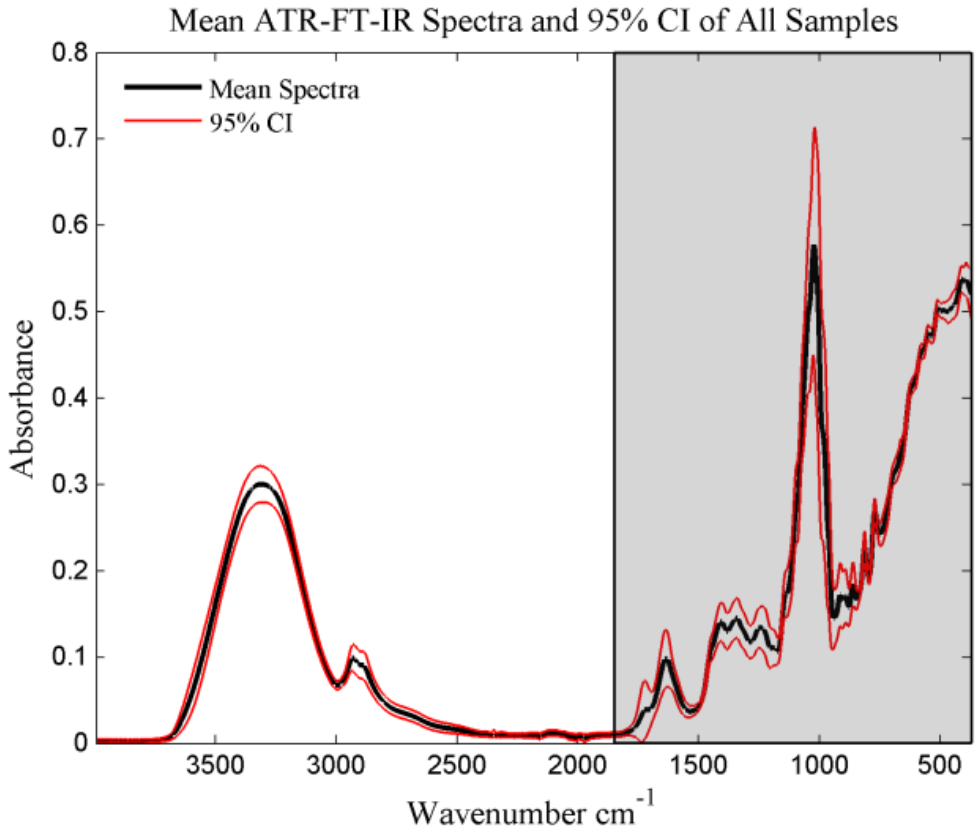


Figure 9: Mean ATR-FT-IR Spectra used for data analysis.

Exploratory data analysis

For the purposes of exploratory data analysis ATR data was converted to absorbance to correct for differences in sample penetration of the beam at different wavelengths. Spectra from all samples and the 95% confidence interval for absorbance was plotted to identify regions of interesting variation. A principal component analysis of the standard normal variates (SNV) scaled and mean centred data between the regions 375-1850 cm^{-1} was conducted using the singular value decomposition (SVD) algorithm with principal components (PC) 1-2 and plotted to identify specific sample groupings based on pathogen and sampling time. Inspection of the sample scores for date and pathogen reveal a cluster of ten samples from the *Penicillium* data set, scanned sequentially, that are largely separate from the remaining samples. This group of samples appears to be separate due to a poor background scan prior to the analysis of the ten samples. For the purposes of cluster analysis and development of predictive modelling based upon pathogen, models were constructed with these samples retained and excluded. As the models with excluded samples did not have greater predictive quality, the results are presented below in Figure 10 for models with all samples and this serves as an indirect indicator of the robustness of the modelling.

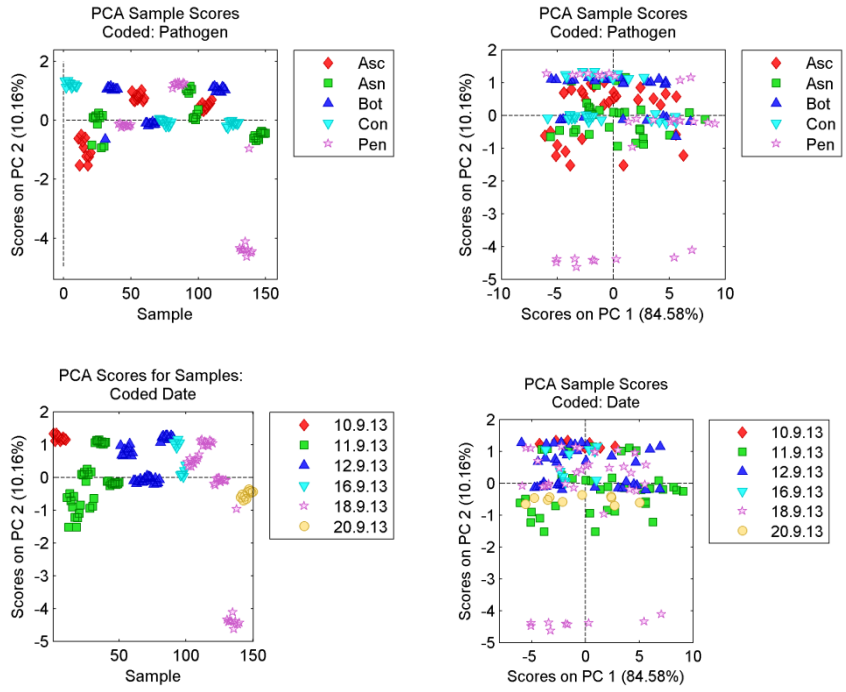


Figure 10: PCA of Samples with coding for date of analysis and berry pathogen.

Development of predictive models of pathogen

Prior to the development of predictive models of pathogen samples were randomly assigned to either a training or test set such that the distribution of samples was approximately similar. The distribution of samples is shown in Table 8.

Table 8: Sample allocation to training and test data sets.

Pathogen/ Treatment	Identity	Training Set		Independent Test Set		TOTAL	
		Number Samples	Percent	Number Samples	Percent	Number Samples	Percent
Control	CON	23	21.9	7	20.0	30	21.4
Aspergillus carbonarius	ASC	22	21.0	8	22.9	30	21.4
Aspergillus niger	ASN	23	21.9	7	20.0	30	21.4
Botrytis cinerea	BOT	23	21.9	7	20.0	30	21.4
Penicillium expansum	PEN	14	13.3	6	17.1	20	14.3
TOTAL		105	100.0	35	100.0	140	100.0

Predictive models of class based upon the pathogen were constructed using the classification modelling algorithms: Soft independent modelling of class analogy (SIMCA); Support Vector Classification (SVR), K-nearest neighbour (KNN) and a machine learning classification approach termed Random Forest which comprises an ensemble of decision trees constructed with the Adaboost method using the raw data. SIMCA, SVR and KNN models were constructed on the infrared spectra between 375-1850cm^{-1} using SNV and mean centred data in the PLS Toolbox version 7.8 (Eigenvector) and random forest ensembles on the same spectral region using the raw data using the Matlab (The Mathworks) version 7.14 Statistical Toolbox. Individual class models for SIMCA were constructed using principal components analysis retaining between three to six principal components for each class. The number of retained PCs was determined by inspection of the data variance explained for each PC, and the size of the eigenvalues associated with each PC. The SVR, KNN and Random Forest models were cross validated using a 10 fold data split of the training data set. The predictive acuity of each model was assessed using the independent test data set and model comparisons for KNN,SVM, SIMCA and Random Forest models are presented as Receiver Operating Characteristic curves that illustrate False Positive versus True Positive class predictions based upon the independent data set.

ROC curves are illustrated in Figure 11 and a theoretical ROC curve that illustrates perfect and random classification assignment is shown in Figure 12 for comparison. Confusion tables and matrices are appended to the document.

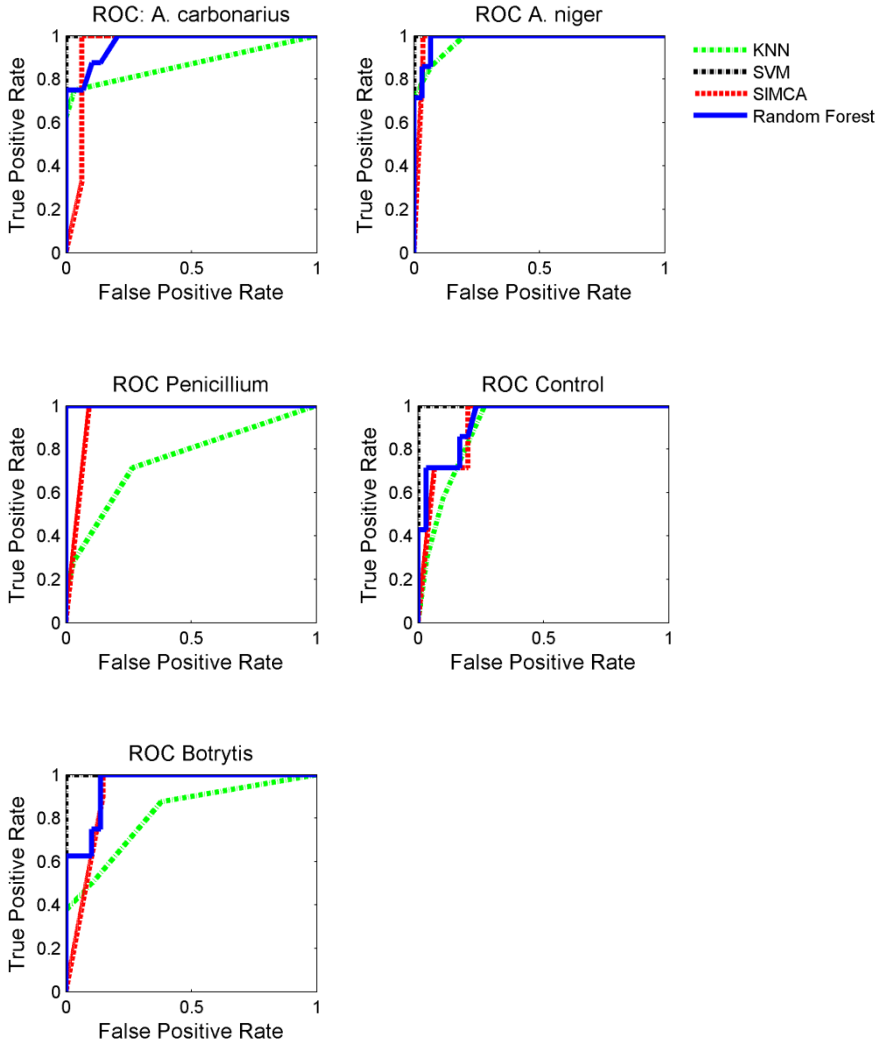


Figure 11: Receiver Operating Characteristic Curve for prediction of Aspergillus carbonarius, Aspergillus niger, Penicillium expansum *and* Botrytis cinerea *infected and differentiation from control (uninoculated) berries using four classifying models.*

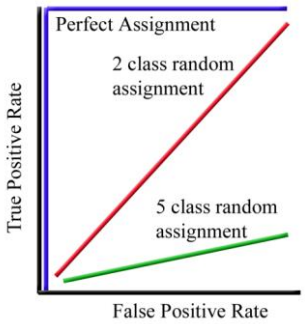


Figure 12: Example of Receiver Operating Characteristic (ROC) curves for perfect and random assignment of predicted class for binary and five class models.

Promising results were obtained using Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FT-IR). ATR-FT-IR is a technique that involves the collection of infrared spectra of samples using reflectance spectroscopy. ATR-FT-IR techniques offer a rapid analytical approach for the measurement of sample composition when the spectrum is applied to previously developed chemometric models. Bunch rot pathogens impart changes in the composition of berries during growth and these changes may elicit infrared signals that can be used to differentiate pathogen and infection stage.

In this preliminary series of experiments an increased level of sensitivity for ATR-FT-IR was developed by using small quantities of grape berry extract which were concentrated *in-situ* on the ATR surface prior to spectral scanning. A comparison of sample scans at room temperature and 60°C is illustrated in Figure 7. A compromise between increasing analytical sensitivity and sample throughput was considered important hence the final method incorporated a sample 'concentrating' time of 5 minutes at 60°C on the ATR surface (Figure 10). Reproducibility of this method was determined using a single sample analysed 10 times with results considered appropriate for a pilot study (data not shown).

Scans of infected and control berry juice that had been frozen and thawed were taken over the course of approximately one week with a background scan every 10 samples. The fingerprint region of the IR spectrum was chosen for data analysis (Figure 9) and exploratory data analysis by PCA of the IR spectra indicated that a subsample of the 150 berry samples was affected by a poor background correction (Figure 10). These samples were a contiguous block of samples of *Penicillium* infected berries and for the purposes of modelling these samples were retained within the four classification models. Retaining these samples offers an alternative comparison of the robustness of the modelling in addition to typical model performance indicators such as sensitivity and specificity measures.

Receiver Operating Characteristic (ROC) curves by definition show true positive rate versus false positive rate (equivalently, sensitivity versus 1–specificity) for different thresholds of the various predictive models. When assessing various predictive models of class (berry pathogen) using independent tests set data, i.e. samples that were not used to construct the predictive model but which class membership is known, overall model performance can be assessed and compared. The ROC curves can be used to find the threshold that maximizes the classification accuracy or to assess, in more broad terms, how the classifier performs in the regions of high sensitivity and high specificity.

Support vector models (SVM) show excellent class prediction for all pathogens and control samples and should be explored further to determine potential for use with large samples and increased grape varieties. SVM models are non-linear and employ a mathematical

transformation of data into a linear subspace so that linear data modelling can be employed. These are computationally intensive models but deserve a more thorough investigation for potential use for classification of bunch rot pathogens.

Random forest models may also be useful for further exploration as there is the possibility to incorporate binary and non-numerical data within the classifiers, for example, grape cultivar, region, visual observation of vine and berry health. Random forest models performed well for classification of samples infected with *Aspergillus* spp. to the species level, although imperfect prediction of class is noted. When predicted class was collapsed to the genus level, i.e. removal of species level information the predictive model performance is improved with 100% true positive and true negative classification for random forest and SVM models (data from confusion matrix in Appendix 5). The SIMCA models were also improved for classification of Aspergillus genus but with a small decrease in predictive performance for true negative (false positive) prediction for the Aspergillus genus.

Prediction of *Penicillium* infected berries and control samples showed mixed results. Possible model performance for prediction of *Penicillium* is impacted by inclusion of outlier samples as previously discussed with SIMCA and KNN models performing with decreased acuity than other models. Similar observations for class prediction are evident for *Botrytis* infected berries for SIMCA and SVM models; with KNN models performing poorly.

6. Outcome/Conclusion:

Fungal rots of grapes can be caused by a number of different berry rotting organisms. Often the individual fungi involved are hard to differentiate, yet the impact they have on grape and wine quality varies. The project has explored different methods that could potentially be used to provide a rapid estimate of bunch rot type and severity.

Laccase production varied depending on the culture conditions of the fungal isolate which limits the use of laccase as an indicator of bunch rot contamination. Glycerol : Gluconic acid ratios were lower in the two species of Aspergillus investigated than in the isolates of *B. cinerea* and *P. expansum* investigated. However, no firm conclusion can be drawn in the absence of data on the fungal biomass present in the material examined.

A. carbonarius, A. niger, B. cinerea and *P. expansum* all produced a similar range of compounds associated with off flavours and aromas when inoculated on to Chardonnay grape berries, although there were differences in the relative proportions of these compounds. It is unlikely that measurements of these compounds can be used as a tool to differentiate between the different types of fungi involved in bunch rot of grapes, but rather these measurements will assist the research in terms of setting thresholds for bunch rot contamination of fruit before fruit is rejected by wineries.

ATR-FT-IR was able to distinguish individual berries inoculated in the laboratory with pure cultures of each of the fungal pathogens examined in this study. These preliminary experiments illustrate the potential of the application of ATR-FT-IR coupled with chemometrics to develop predictive models of bunch rot pathogens. Further work is required that incorporates larger sample sets and varying rates of infection which are normalised for fungal biomass. The technique will also have to be evaluated using field examples of naturally occurring bunch rots which contain a mixture of fungal pathogens. This work is on-going with a substantially expended data set currently under investigation. Further work in which different grape cultivars are added to the models will also be of interest for creating a useful industry useable model.

Two methods of estimating fungal biomass from infected vine tissues were investigated. Possible interference from contaminating compounds limited the scope of using chitin as an indicator of fungal biomass. More promising results were obtained with measurements of the fungal sterol, ergosterol. It is anticipated that in future experiments ergosterol content will be

used as a quantitative measurement of fungal biomass. This will allow for a more accurate comparison of methods for determining bunch rot contamination of grapes and wine.

In conclusion, this project achieved the goal to obtain preliminary data on techniques for quantifying and differentiating bunch rot diseases of grapes. The results from this pilot project have formed a foundation for on-going research (AGWA project NWG 14-02).

7. Recommendations

The work described in this report is on-going and will be continued in AGWA project NWG 14 -02. The current project utilised three isolates of *B. cinerea*, two species of Aspergillus and one isolate of *P. expansum*. Fungi are genetically diverse and adaptable to changing environmental conditions. Further validation of the research outcomes described in this report will need to be completed with a wider range of fungal isolates and species. Much of the work in this pilot project has relied on detached individual berries rather than whole bunches and has involved just one wine grape cultivar, Chardonnay. Future research will examine field-grown bunches in more detail and will utilise a wider range of wine grape cultivars. Ergosterol will be used as an indicator of total fungal biomass in bunch rot affected bunches, and measurements will be expressed with respect to ergosterol fungal biomass equivalents. The work will also be extended to look more closely at the impact bunch rots have on wine quality and what practices can be employed in the wine production process to ameliorate wine faults. It should then be possible to more fully identify bunch rot thresholds for wine contamination.

Appendix 1: Communication

Industry presentations

Date	Presentation	Event / location	Presenters	Approx. Number of attendees
29th May 2013	Bunch rot identification, biology and management	Hastings Valley Vignerons Assoc., Wauchope, NSW	C. Steel	15
15th Jul 2013	What rot is that?	Workshop 29, 15th Australian Wine Industry Technical Conference. Sydney	C. Steel	25
15th Jul 2013	New tools: immuno-detection, off-flavours, ATR-FTIR	Workshop 29, 15th Australian Wine Industry Technical Conference. Sydney	N. Sadoughi C. Steel	25
11th Oct 2013	What a lot of rot: The latest in bunch rot research	Canberra International Riesling Challenge, Canberra Hyatt, ACT	C. Steel	40
24th Oct 2013	Management and biology of bunch rot diseases of grapes	Wine Industry Cluster /AWRI seminar series, Adelaide, SA	C. Steel	15
27th May 2014	The latest developments for bunch rot detection and differentiation	Hunter Valley, NSW	C. Steel L. Schmidtke N. Sadoughi	15
31st Jul 2014	The latest developments for bunch rot detection and differentiation	Roberts Viticulture Expo, The Grange Function Centre Campbell Town, TAS	C. Steel L. Schmidtke	70
4th Aug 2014	Botrytis biology and best practice management	Viticultural Society of the Canberra District, Murrumbateman, NSW	C. Steel	25

Conference proceedings

Sadoughi, S, Schmidtke, L M, Blackman, J W, Steel C C and Boissery, K A (2014) Improved headspace GC-MS analysis of geosmin in grey mould affected wine, Proc 15th Australian Wine Industry Technical Conference. 13 – 18th July 2013. Sydney, Australia. Abstract 163, pp276 - 277. Eds: Beames, K, Robinson, E, Godden, P and Johnson, D.

Fact sheet

Steel, C C (2014) Non-Botrytis bunch rots: Questions and Answers. GWRDC Fact sheet. http://research.agwa.net.au/wp-content/uploads/2014/07/Non-botrytis-bunch-rots-Final.pdf

Media presentation

ABC Rural Radio Mid and North Coast NSW interview 11th July 2014 Stopping rots in sub-tropical vineyards. http://www.abc.net.au/news/2014-07-21/wine-rot-research-sub-tropical/5611592

Appendix 2: Intellectual Property*:*

Identify the intellectual property and/or valuable information arising from the research.

Methods used in this project are available in the public domain, our application of these methods is on-going, intellectual property will be further evaluated in the future funded project, NWG 14-02.

Appendix 3: References

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Diaz, G.A., L. Yanez, and B.A. Latorre (2011) Low occurrence of patulin-producing strains of Penicillium in grapes and patulin degradation during winemaking in Chile. American Journal of Enology and Viticulture 62, 542-546. doi: 10.5344/ajev.2011.11034.

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Appendix 5:

1. Off flavours identified in inoculated grapes by GC/MS and internal standards used.

Table 9a: Details of off flavour compounds identified from inoculated grape berry samples and their corresponding odours

Compound	Retention time (min)	Ion m/z	Odour
1-Octen-3-one	16.9	55, 70,83, 97	Fungus [a]
1-Octen-3-ol	21.1	57, 72, 85, 99	Mushroom Fungus [a]
2-Methylisoborneol (MIB)	25.8	107, 135, 95	Muddy[a]
Geosmin	33.5	112, 111, 125	Muddy[a]

Table 9b: Details of internal standards used for GC/MS analysis of off flavour compounds

Compound	Retention time (min)	Ion m/z
Methyl-isobutyl-ketone	9.5	58, 85, 100
2-Octanol	21.3	45, 55, 56, 70
2-Methyl-D3-isoborneol	26.0	110, 138,55
Geosmin-D3	34.4	115,129,128

[a]: (Boutou and Chatonnet 2007)

2. Chromatograms for ergosterol standard

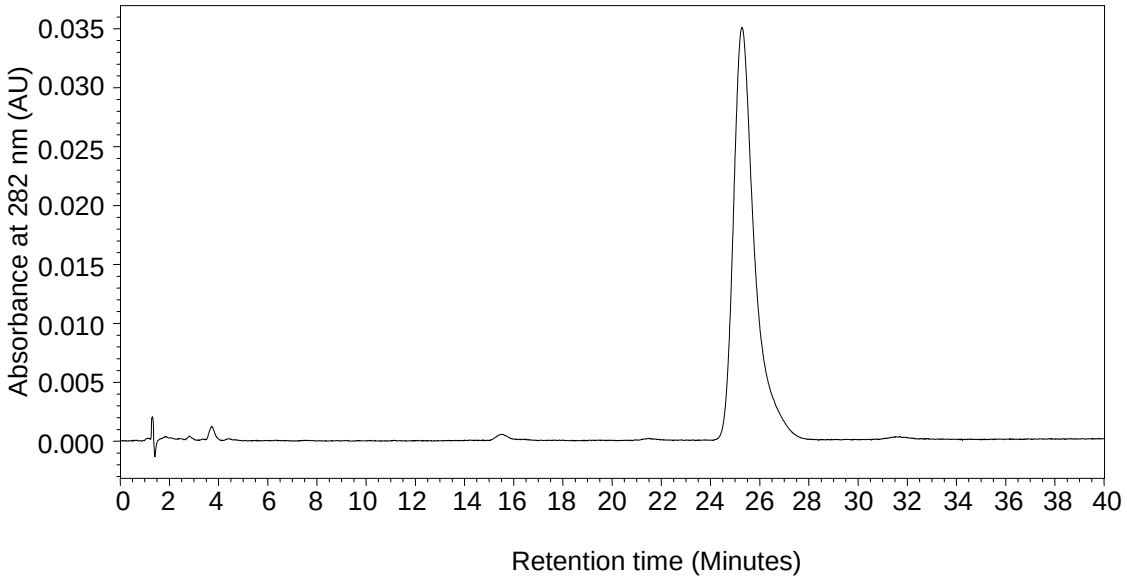


Figure 13: Typical chromatogram for ergosterol standard

3. ATR-FT-IR Raw data

Confusion Tables for Model Data

FTIR BUNCH ROT CLASSIFICATION USING ALL SAMPLES

RANDOM FOREST ADABOOST METHOD

Cross Validation of Training Data Set

Table 10. Confusion Matrix for cross validation using the training sample data set.

Class:	True Positive	False Positive	True Negative	False Negative
Asc	1.00000	0.00000	1.00000	0.00000
Asn	0.95455	0.02198	0.97802	0.04546
Bot	0.60000	0.07955	0.92046	0.40000
Con	0.59259	0.08140	0.91860	0.40741
Pen	1.00000	0.06250	0.93750	0.00000

Table 11. Confusion Table for cross validation using the training sample data set.

	Actual Class				
Class:	Asc	Asn	Bot	Con	Pen
Predicted as Asc	22	0	0	0	0
Predicted as Asn	0	21	1	1	0
Predicted as Bot	0	0	15	7	0
Predicted as Con	0	0	7	16	0
Predicted as Pen	0	1	2	3	17

Prediction of Class using the Independent Data Set

Table 12. Confusion Matrix for prediction of the independent data set.

Class:	True Positive	False Positive	True Negative	False Negative
Asc	1.00000	0.09375	0.90625	0.00000
Asn	0.70000	0.00000	1.00000	0.30000
Bot	0.50000	0.00000	1.00000	0.50000
Con	1.00000	0.14286	0.85714	0.00000
Pen	1.00000	0.09091	0.90909	0.00000

Table 13. Confusion Table for prediction of the independent data set

	Actual Class				
Class:	Asc	Asn	Bot	Con	Pen
Predicted as Asc	5	3	0	0	0
Predicted as Asn	0	7	0	0	0
Predicted as Bot	0	0	8	0	0
Predicted as Con	0	0	5	2	0
Predicted as Pen	0	0	3	0	4

KNN Model ALL SAMPLES

Training Samples Model Results

Table 14. Confusion matrix for training samples KNN. The confusion matrix shows the proportion of training samples correctly and incorrectly assigned to their class.

Class:	True Positive	False Positive	True Negative	False Negative
Asc	0.90909	0.02198	0.97802	0.09091
Asn	0.65217	0.07778	0.92222	0.34783
Bot	0.63636	0.17582	0.82418	0.36364
Con	0.47826	0.10000	0.90000	0.52174
Pen	0.60870	0.05556	0.94444	0.39130

Table 15. Confusion table for the training samples shows the numbers of correctly assigned samples according to their class.

	Actual Class				
Class:	Asc	Asn	Bot	Con	Pen
Predicted as Asc	20	2	0	0	0
Predicted as Asn	2	15	1	2	2
Predicted as Bot	0	5	14	8	3
Predicted as Con	0	0	5	11	4
Predicted as Pen	0	1	2	2	14

Cross Validation of Training Data Set

Table 16. Confusion Matrix for cross validation using the training sample data set.

Class:	True Positive	False Positive	True Negative	False Negative
Asc	0.86364	0.01099	0.98901	0.13636
Asn	0.65217	0.05556	0.94444	0.34783
Bot	0.54545	0.15385	0.84615	0.45455
Con	0.56522	0.11111	0.88889	0.43478
Pen	0.60870	0.11111	0.88889	0.39130

Table 17. Confusion Table for cross validation using the training sample data set.

	Actual Class				
Class:	Asc	Asn	Bot	Con	Pen
Predicted as Asc	19	1	0	0	0
Predicted as Asn	3	15	0	1	1
Predicted as Bot	0	5	12	5	4
Predicted as Con	0	0	6	13	4
Predicted as Pen	0	2	4	4	14

Prediction of Class using the Independent Data Set

Table 18. Confusion Matrix for prediction of the independent data set.

Class:	True Positive	False Positive	True Negative	False Negative
Asc	0.62500	0.00000	1.00000	0.37500
Asn	1.00000	0.10000	0.90000	0.00000
Bot	0.62500	0.24138	0.75862	0.37500
Con	0.57143	0.10000	0.90000	0.42857
Pen	0.28571	0.03333	0.96667	0.71429

Table 19. Confusion Table for prediction of the independent data set

	Actual Class				
Class:	Asc	Asn	Bot	Con	Pen
Predicted as Asc	5	0	0	0	0
Predicted as Asn	2	7	1	0	0
Predicted as Bot	1	0	5	2	4
Predicted as Con	0	0	2	4	1
Predicted as Pen	0	0	0	1	2

SVM Classification

Table 20. Confusion matrix for training samples XXX. The confusion matrix shows the proportion of training samples correctly and incorrectly assigned to their class.

Class:	True Positive	False Positive	True Negative	False Negative
Asc	1.00000	0.00000	1.00000	0.00000
Asn	1.00000	0.00000	1.00000	0.00000
Bot	0.95455	0.01099	0.98901	0.04545
Con	0.95652	0.01111	0.98889	0.04348
Pen	1.00000	0.00000	1.00000	0.00000

Table 21. Confusion table for the training samples shows the numbers of correctly assigned samples according to their class

	Actual Class				
Class:	Asc	Asn	Bot	Con	Pen
Predicted as Asc	22	0	0	0	0
Predicted as Asn	0	23	0	0	0
Predicted as Bot	0	0	21	1	0
Predicted as Con	0	0	1	22	0
Predicted as Pen	0	0	0	0	23

Cross Validation of Training Data Set

Table 22. Confusion Matrix for cross validation using the training sample data set.

Class:	True Positive	False Positive	True Negative	False Negative
Asc	0.86364	0.01099	0.98901	0.13636
Asn	0.78261	0.03333	0.96667	0.21739
Bot	0.54545	0.14286	0.85714	0.45455
Con	0.60870	0.15556	0.84444	0.39130
Pen	0.73913	0.02222	0.97778	0.26087

Table 23. Confusion Table for cross validation using the training sample data set.

	Actual Class				
Class:	Asc	Asn	Bot	Con	Pen
Predicted as Asc	19	1	0	0	0
Predicted as Asn	3	18	0	0	0
Predicted as Bot	0	3	12	8	2
Predicted as Con	0	1	9	14	4
Predicted as Pen	0	0	1	1	17

Prediction of Class using the Independent Data Set

Table 24. Confusion Matrix for prediction of the independent data set.

Class:	True Positive	False Positive	True Negative	False Negative
Asc	0.75000	0.00000	1.00000	0.25000
Asn	1.00000	0.06667	0.93333	0.00000
Bot	1.00000	0.06897	0.93103	0.00000
Con	1.00000	0.00000	1.00000	0.00000
Pen	0.71429	0.00000	1.00000	0.28571

Table 25. Confusion Table for prediction of the independent data set

	Actual Class				
Class:	Asc	Asn	Bot	Con	Pen
Predicted as Asc	6	0	0	0	0
Predicted as Asn	2	7	0	0	0
Predicted as Bot	0	0	8	0	2
Predicted as Con	0	0	0	7	0
Predicted as Pen	0	0	0	0	5

SIMCA ALL SAMPLES SNV MC

Table 26. Confusion matrix for training samples XXX. The confusion matrix shows the proportion of training samples correctly and incorrectly assigned to their class.

Class:	True Positive	False Positive	True Negative	False Negative
Asc	1.00000	0.02198	0.97802	0.00000
Asn	0.91304	0.17778	0.82222	0.08696
Bot	0.59091	0.04396	0.95604	0.40909
Con	0.56522	0.00000	1.00000	0.43478
Pen	0.95652	0.00000	1.00000	0.04348

Table 27. Confusion table for the training samples shows the numbers of correctly assigned samples according to their class

	Actual Class				
Class:	Asc	Asn	Bot	Con	Pen
Predicted as Asc	22	2	0	0	0
Predicted as Asn	0	21	9	6	1
Predicted as Bot	0	0	13	4	0
Predicted as Con	0	0	0	13	0
Predicted as Pen	0	0	0	0	22

Prediction of Class using the Independent Data Set

Table 28. Confusion Matrix for prediction of the independent data set.

Class:	True Positive	False Positive	True Negative	False Negative
Asc	0.75000	0.06897	0.93103	0.25000
Asn	0.71429	0.10000	0.90000	0.28571
Bot	0.87500	0.20690	0.79310	0.12500
Con	0.42857	0.00000	1.00000	0.57143
Pen	0.71429	0.00000	1.00000	0.28571

Table 29. Confusion Table for prediction of the independent data set

	Actual Class				
Class:	Asc	Asn	Bot	Con	Pen
Predicted as Asc	6	2	0	0	0
Predicted as Asn	2	5	1	0	0
Predicted as Bot	0	0	7	4	2
Predicted as Con	0	0	0	3	0
Predicted as Pen	0	0	0	0	5