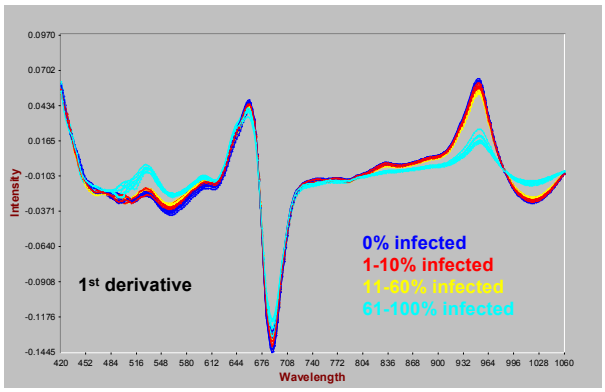
THE UNIVERSITY OF ADELAIDE AUSTRALIA

HARDY WINE COMPANY

Australian Government
Grape and Wine Research and Development Corporation

Application of NIR for disease assessment





FINAL REPORT to GWRDC

GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

Project Number: **UA 05/08**

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1. Abstract

Chardonnay grapes with varying degrees of powdery mildew were collected by industry viticultural staff in two different growing regions. Grape samples were homogenised, analysed for powdery mildew DNA content, and scanned by reflectance spectroscopy, over a

wavelength range covering the visible and near infrared (NIR) regions of the electromagnetic spectrum. The powdery mildew DNA content and spectral information generally correlated with the visual infection classification, except that disease severity, as determined by visual inspection, had been overestimated at one sampling site. The visual infection grading and powdery mildew DNA content could be predicted from the spectral information using discriminant analysis and partial least squares (PLS) regression, respectively.

2. Executive summary

This project was established in consultation with the Hardy Wine Company. The Hardy Wine Company currently uses Near Infra-red Reflectance Spectroscopy (NIR) for some standard quality tests at the winery and is keen to expand the instrument capabilities to include rapid disease assessment. Application of the NIR method would provide the first practical means of quantifying powdery mildew in grape loads in real time. Such a method should reduce disputes between grapegrowers and wineries, such as have occurred as a result of the current practice of visual assessment of fungal contamination. It is also likely that the introduction of a practical, quantitative method for the assessment of powdery mildew would lead to the establishment of new thresholds for the disease within grape contracts that better reflect the detrimental effect of the fungus on wine quality.

The objectives of the project were to: 1) capitalise on existing knowledge, expertise and research tools developed in CRV 99/23 (CRCV Project 1.5.2); 2) test the NIR protocol for popular varieties of red and white grapes sourced from major wine grape growing regions of South Australia, from both warm and cool climate; 3) test the technology with leading industry collaborators.

Summary of R&D outcomes

The suitability of NIR for estimating powdery mildew in grapes collected by staff of the Hardy Wine Company in vintage 2006 was assessed. Data were collected for comparison of NIR, visual and DNA-based disease assessments. Two sample preparation methods (mechanical homogenisation and crushing by hand) and two DNA extraction methods (CTAB and commercial kit) were compared also. Chardonnay grape samples collected from two regions, Berri Estates, Riverland, and Tintara, McLaren Vale, were processed. There was insufficient powdery mildew on other varieties to warrant collection. Industry personnel sorted grapes into five categories (0%, <5%, 5-10%, 10-30% and >30%) according to percentage of the bunch with powdery mildew. Grapes were frozen and 62 samples were sent to Waite Campus, homogenised and subdivided for DNA and NIR analysis.

The amount of fungal DNA recovered was similar for both extraction methods (Stummer et al. 2006). While DNA content determined following mechanical homogenization was generally less than that determined following crushing by hand, *E. necator* DNA content and powdery mildew severity were positively correlated using both sample preparation methods. Likewise, when samples from a given region were compared, *E. necator* DNA content generally increased with increasing powdery mildew severity, with one exception. There was a considerable difference between the two regions in terms of the amount of fungal DNA in grape samples considered to have >30% powdery mildew. The large range in the severely diseased category (30-100% of the bunch affected) resulted in a large spread of DNA values, however, the amount of *E. necator* DNA detected in samples from Tintara indicated that

disease severity, as determined by visual inspection, had been overestimated. The data support the assumption that assessing powdery mildew severity visually is subjective and varies between assessors.

The homogenised grape samples were scanned by reflectance spectroscopy, over a wavelength range covering the visible and NIR regions of the electromagnetic spectrum. Principal component analysis (PCA) showed strong clustering of samples correlating with infection level. The visual infection grading and powdery mildew DNA content from an individual site could be predicted from the spectral information using discriminant analysis and partial least squares (PLS) regression, respectively. The prediction rate was reduced with combined sets of samples from the different sites but the highest error was in the form of false positives rather than false negatives. The powdery mildew DNA content and spectral information correlated with the visual infection classification, although there appeared to be differences between the sampling sites. A limited number of Botrytis-affected grape samples were collected from one site in vintage 2006. However, these samples were not visually categorised for degree of infection and, as disease severity cannot be assessed after freezing, they were not analysed further.

DNA data proved to be a more reliable reference point for NIR than did visual assessment of the severity of powdery mildew on bunches.

Viticultural staff of the Hardy Wine Company collected diseased samples in vintage 2006. The project would not have been possible without their support. The project budget was sufficient only for analysis of samples collected from the 2006 vintage. As such, no samples from the 2007 vintage were collected for this project. Future work is warranted for other white and red varieties and for initial NIR calibration using industry facilities.

3. Background

Powdery mildew, caused by the fungus *Erysiphe necator* (formerly *Uncinula necator*), is an economically important disease of grapevines world-wide. Powdery mildew on grapes can adversely affect wine quality (Ough and Berg 1979, Pool et al. 1984, Amati et al. 1996, Gadoury et al. 2001, Darriet et al. 2002, Stummer et al. 2003 and 2005). Wines made from powdery mildew-affected grapes have negative sensory attributes (Stummer et al. 2003 and 2005). Stummer et al. (2003, 2005) showed that Chardonnay wines made from grapes with as little as 1-5 % of bunches affected by powdery mildew had greater phenolic content and were perceived by a sensory panel as having more pronounced viscous/oily mouth feel than wines made from disease-free grapes.

Botrytis cinerea is the primary cause of bunch rot in grapes and infection in harvested grapes impacts negatively on wine quality through oxidative reactions caused by fungal laccase and soluble fungal polysaccharides which lead to problems in clarification (Girbau et al. 2004). Both *B. cinerea* and *E. necator* are often associated with secondary infection by other microorganisms which can introduce off-flavours into the wine.

Both powdery mildew and botrytis bunch rot diseases are difficult to detect and assess visually in machine-harvested loads delivered in bins or in trucks. Weighbridge assessment of grapes for powdery mildew is currently subjective and semi-quantitative, and yet the result is used by wineries to reduce the payment for 'diseased' fruit. An objective, quantitative method of assessment that allows repeat measures would provide a more reliable result on which to base prices. The method could also be used by grapegrowers to evaluate the effectiveness of management strategies and, coupled with information on what levels of infection are acceptable, could be used to assess whether or not fruit meets quality specifications. Powdery mildew is also very difficult to detect on red grapes, due to the camouflaging effect of the dark skin colour on the berry infection. There is therefore a need for a rapid, objective method to detect and quantify disease in grape consignments.

Near infrared spectroscopy has become the analysis method of choice in food, agriculture and pharmaceutical industries where large throughput with minimal sample preparation is required. In the wine industry, the predominant use of NIR methods to date has been in the analysis of wine ethanol (Dambergs et al. 2004), and it is also applicable in the analysis of methanol in wine fortifying spirit (Dambergs et al. 2002). Methods for simultaneous analysis of grape quality parameters such as total soluble solids, pH and anthocyanins have also been developed (Gishen and Dambergs 1998, Dambergs et al. 2003, 2006, Cozzolino et al. 2004, 2006). Some large wine companies are using this technology to measure grape anthocyanins on a routine basis (Kennedy, 2002) and it may be possible to use the same methods to simultaneously monitor the degree of mould contamination. The use of NIR for detecting fungal contamination has been demonstrated for powdery mildew and rust in grain (Asher et al. 1982) and for mould contamination of tomato puree (Davies et al. 1987). We performed preliminary studies (CRV 99/23 (CRCV Project 1.5.2)) with samples from trial plots to demonstrate the ability to detect powdery mildew in grapes with NIR spectroscopy (Dambergs et al. 2004, Scott et al. 2006).

As part of CRV 99/23 (CRCV Project 1.5.2), DNA-based probes have been developed to quantify powdery mildew in berries, must and juice (Stummer and Scott 2000, Stummer et al. 2002). There was a strong correlation between powdery mildew on berries assessed by visual

inspection (aided by microscopy) and amount of powdery mildew DNA detected using the powdery mildew specific DNA probe (Stummer et al. 2006).

Using samples selected visually, and confirmed with DNA analysis, preliminary work with Chardonnay grapes has demonstrated that NIR spectroscopy can discriminate the lowest powdery mildew category (1-5% infected) from uninfected samples. Although there may be slight overlap with adjacent categories, NIR can also discriminate between infection categories and this discrimination was related primarily to the fungus, not based on co-linearity with differences in total soluble solids or pH that are often induced by fungal infection (Dambergs et al. 2005). Importantly, strong correlation was shown between NIR and DNA data.

In preliminary experiments using Shiraz grapes that had been puncture-inoculated with *B. cinerea* in the laboratory, NIR detected infection; batches with 2% of grapes showing grey mould (sporulation) could be discriminated from the uninoculated control (AWR03/03IDS: WT&P).

Information generated using NIR and DNA data in CRV 99/23 (CRCV Project 1.5.2) was obtained from a small number of trial plots, mostly with Chardonnay grapes from the Adelaide Hills and following visual and microscopic assessment of disease by expert plant pathologists. To determine the suitability of NIR for industry use as an objective measurement of powdery mildew in grapes, it was necessary that industry personnel collect diseased grape samples from other varieties and from other regions.

4. Project Aims and Performance targets

Project Aims

- Capitalise on existing knowledge, expertise and research tools developed in CRV 99/23 (CRCV Project 1.5.2).
- Test NIR protocol for popular varieties of red and white grapes sourced from the major winegrape growing regions of South Australia, from both warm and cool climate.
- Technology tested by leading industry collaborators.

Performance targets and outputs

Outputs and Performance Targets 2005-06

Outputs	Performance Targets
1. Preliminary information on the correlation between disease severity, content of powdery mildew DNA and NIR spectra for samples from a range of viticultural regions and grape varieties.	Begin evaluation of NIR with a large sample range as a tool for rapid assessment of powdery mildew contamination at the weighbridge.

Outputs and Performance Targets 2006-07

Outputs	Performance Targets
1. Information on the correlation between disease severity, content of powdery mildew DNA and NIR spectra on samples from a range of viticultural regions and grape varieties.	Evaluation of NIR with a large sample range as a tool for rapid assessment of powdery mildew contamination at the weighbridge.
2. Validate NIR as a means of quantification of powdery mildew and Botrytis in an industry setting, in-house at Hardy Wine Company and others that have NIR facilities	NIR evaluated for quantification of powdery mildew and Botrytis in an industry setting.
3. Communication of correlation between NIR and DNA to industry	Results of research presented at workshops and submitted for scientific and industry publication.

Samples were obtained for Chardonnay only as there was insufficient powdery mildew on other varieties to warrant collection. Likewise, there were insuffient grapes samples (of any variety) that were clearly classified with degree of Botrytis bunch rot to warrant NIR analysis.

The final project application was approved with a significantly reduced budget, which meant that not all of the original Outputs and Performance Targets 2006-07 could be achieved. There were insufficient funds to validate NIR in-house by industry, to conduct DNA analysis of samples collected in vintage 2007 or to develop and test a quantitative PCR-based assay that could be provided as a diagnostic service. A quantitative PCR test would be cheaper and more rapid than the existing slot assay and would not involve radioactivity.

5. Methods

Sample collection
Chardonnay grape samples were collected by Hardy Wine Company viticultural staff in the Riverland (Berri Estates) and McLaren Vale (Tintara) regions. The samples were visually classified for severity of powdery mildew using a key based on the percentage of the surface area of the grape bunch affected by *E. necator* (R. Emmett and T. Wicks pers. com., 2000). Industry collaborators classified grape bunches into five categories; A = healthy - 0%, B = <5%, C = 5-10%, D = 10-30% and E = >30% of the bunch surface with powdery mildew, froze them and sent them to the Waite Campus for analysis.

Sample preparation
62 grape samples (approximately 250 g each) were thawed then homogenized while still cool (approximately 4°C) with a *Grindomix GM 200* (Retsch GmbH & Co., Haan, Germany) for 20 seconds at 8,000 rpm using a floating lid to maintain contact of sample with the blades. Homogenates were immediately sub-sampled (10 ml) and frozen for later DNA analysis while the remainder of the homogenate was used in NIR analysis.

In addition, the effect of method of sample preparation on quantification of *E. necator* DNA content was determined using data collected in 2002-2004 (CRV 99/23, CRCV 1.5.2). In vintages 2002-2004, grape samples, representing similar disease severity categories, had been prepared by a) crushing 200 berries in a bag by hand, and b) homogenizing grapes in a Grindomix blender (as above), then the amount of *E. necator* DNA was estimated. Data analysis is described below.

DNA analysis
10 ml samples from 2006 vintage were ground to a fine powder in liquid nitrogen and kept at -70°C. DNA was extracted from 1 gram lots using one of two extraction methods as follows: 1) CTAB extraction procedure (Doyle & Doyle 1980), as described by Stummer (et al. 2006); 2) Qiagen Plant DNA extraction kit, as recommended by the manufacturer. The Qiagen kit was tested because it is quick and is routinely used in diagnostic laboratories. Two replicated extractions were performed for each sample and extraction method.

DNA was subjected to electrophoresis and DNA concentration was estimated by comparison with fragments from a λ *Hin*d III- DNA ladder of known concentration (Roche Diagnostics, Mannheim) and confirmed by uv spectrophotometry.

Slot-blot hybridization assays were prepared for all samples using the Bio-dot SF unit (Bio-Rad, Hercules, CA) as recommended by the manufacturer. Membranes were hybridized with the *E. necator*-specific clone, pEnA1, as previously described (Stummer et al. 2006). To quantify powdery mildew DNA present in each grape sample, all slot-blots contained DNA (0.01-5 ng) extracted from conidia of powdery mildew, as a positive control. Signals were quantified by visual comparison of band intensities of a given sample with the band intensity of known amounts of powdery mildew DNA. Slot-blots contained approximately 100 ng of total DNA (grapevine plus powdery mildew).

Statistical analaysis
E. necator DNA data obtained from vintages 2002-2004 (Stummer et al. 2006) using the two methods of sample preparation (homogenization by Grindomix or crushing by hand) were subjected to analysis of variance (ANOVA) using the general linear model. Fisher's least

significant difference means comparison test (P=0.05) was performed to determine statistical significance. *E. necator* DNA data from vintages 2002, 2003, 2004 (Stummer et al. 2006) and 2006 (Table 1, representing a total of four vineyard sites and three viticultural regions) representing similar infection categories, that had been homogenized in a Grindomix blender, were pooled and descriptive statistics generated. Genstat version 6.1.0.200 (Lawes Agricultural Trust, Rothamsted Experimental Station, UK) was used for data analysis.

Table 1. Vineyard location of Chardonnay grape samples.

Year	Location (winery)
2002	Clarendon, Adelaide Hills
2003	Lenswood, Adelaide Hills
2004	Clarendon, Adelaide Hills
2006	Riverland, SA (Berri Estates)
	McLaren Vale, SA (Tintara)

NIR scanning and data analysis

Freshly prepared homogenates were scanned, without temperature equilibration, in a FOSS *NIRSystems 6500* (FOSS NIRSystems, Silver Spring, Maryland, USA), in reflectance mode at 2 nm intervals over the wavelength range of 400–2500 nm, using a 10 mm sample cell and sample transport module. A reference scan was performed before each sample, using a rare earth metal oxide impregnated ceramic tile as a reference. Spectra were stored as the average of 32 scans. Scanning control was performed with the *Vision* software package (FOSS NIRSystems, Silver Spring, Maryland, USA). Spectra were exported as NSAS files for chemometric analysis.

Chemometric analysis was performed with *The Unscrambler* software (Camo Software, Oslo, Norway). Discriminant analysis with principal component analysis (PCA) scores of spectra was performed with *Systat* (SPSS Inc., Chicago USA). Typically, spectra were pre-processed by Savitsgy-Golay smoothing, with first derivative transformation (4 data point gap). Reference data for chemometric analysis were either the visual classification level or the concentration of fungal DNA.

General statistical analysis was performed with *Systat.*

6. Results and discussion

DNA analysis
Similar amounts of total DNA was extracted from homogenized samples using either the Qiagen kit or the CTAB method. The detection threshold and amount of powdery mildew DNA detected using probe pEnA1 were similar irrespective of the extraction method (results not shown).

While DNA content determined following homogenization using the Grindomix mechanical method was generally less than that determined following crushing, *E. necator* DNA content and powdery mildew severity were positively correlated using both sample preparation methods. When data from the three previous vintages were compared, powdery mildew DNA content was significantly less in samples with >30% (2002) and >60% (2003 & 2004) infection following homogenization than by crushing (Table 2). Possible explanations are that mechanical blending physically shears DNA to a greater extent and/or that homogenizing the grape sample liberates more DNA-degrading enzymes into the supernatant than crushing berries by hand. Increasing severity of powdery mildew is associated with an increase in the total grape microbial population (Stummer et al. 2003), and microbes are known to produce DNases and protease enzymes that affect the recovery of DNA. The enzymes are most active at ambient temperatures. Therefore, to minimise degradation of DNA, it is important to process samples quickly and avoid cycles of thawing/freezing.

We have shown previously that *E. necator* DNA content and powdery mildew severity are positively correlated (Stummer et al. 2006). The general trend of increasing fungal DNA concentration with increasing visual symptoms was evident using either homogenization method (Table 2). When grape samples across 4 vintages, with similar disease categories were grouped together, there was a progressive increase in average fungal DNA concentration with increasing visual infection categories from 0 to 30 %, but no further increase for samples with >30% disease (Table 3). The 31-100% disease category also had the largest range of DNA values (0.05-1.5 ng *E. necator* DNA/100 ng total DNA) with the highest max DNA value (1.5 ng) and corresponding large standard deviations (Table 3). The minimum DNA value (0.05 ng) obtained for this disease category was the result of outlying values obtained for Tintara samples in 2006. 0.05 ng *E. necator* DNA/100 ng total DNA is considerably less than the values obtained for equivalent samples in 2002-2004 (Table 2). This discrepancy suggests that assessors overestimated disease severity at Tintara.

Table 2. Mean *Erysiphe necator* DNA content in 5 grams of homogenized Chardonnay grape samples with different severity of powdery mildew, estimated using the slot-blot technique with probe pEnA1, vintages 2002 – 2004. Comparison of two methods of sample preparation, a) 200 berries crushed in bag and b) grape homogenised in a Grindomix blender.

		Powdery mildew disease severity (% of bunch affected) and *E. necator* DNA content (ng/100 ng total DNA)								
Year	Sample	0	1-5	1-10	6-30	11-60	31-60	31-100	61-100	LSD
2002	a	0.00 [a]	0.10 [a b]	-	0.50 [b]	-		1.50 [c]	-	0.40
	b *	0.00 [a]	0.50 [b]	-	0.50 [b]	-		**0.80** [c]	-	0.20
2003	a	0.00 [a]	-	0.05 [a]	-	0.20 [a]		-	0.70 [b]	0.30
	b	0.01 [a]	-	0.01 [a]	-	0.10 [a]		-	**0.40** [b]	0.10
2004	a	0.00 [a]	0.30 [a]	-	0.80 [a]	-	1.80 [b]		2.60 [c]	0.90
	b	0.00 [a]	0.50 [b]	-	0.70 [b]	-	1.00 [c]		**1.00** [c]	0.20

For each vintage (2002, 2003, 2004) and each homogenized must sample, letters in superscript denote significant differences ($P < 0.001$) in *E. necator* DNA content between the powdery mildew severity categories (according to ANOVA and Fisher's Least Significant Difference means comparison test). - indicates not determined. Values in bold show significant differences in *E. necator* DNA content between the equivalent sample preparations within a vintage. 4 replicates per sample except * indicates 3 replicates.

Table 3. Descriptive statistics for *Erysiphe necator* DNA (ng/100 ng total DNA) content of Chardonnay grape samples from 4 vineyard sites and with different severity of powdery mildew, estimated using the slot-blot technique with probe pEnA1. DNA values represent grape samples homogenised in a Grindomix blender from vintages 2002, 2003, 2004 (Stummer et al. 2006) and 2006 with similar infection categories.

Disease severity (%)	No. of samples	*E. necator* DNA content			
		Mean	Std Dev	Min	Max
0	34	0.01	0.02	0.00	0.08
1-5	23	0.20	0.20	0.01	0.50
11-30	11	0.40	0.30	0.10	1.00
31-100	30	0.40	0.43	0.05	1.50

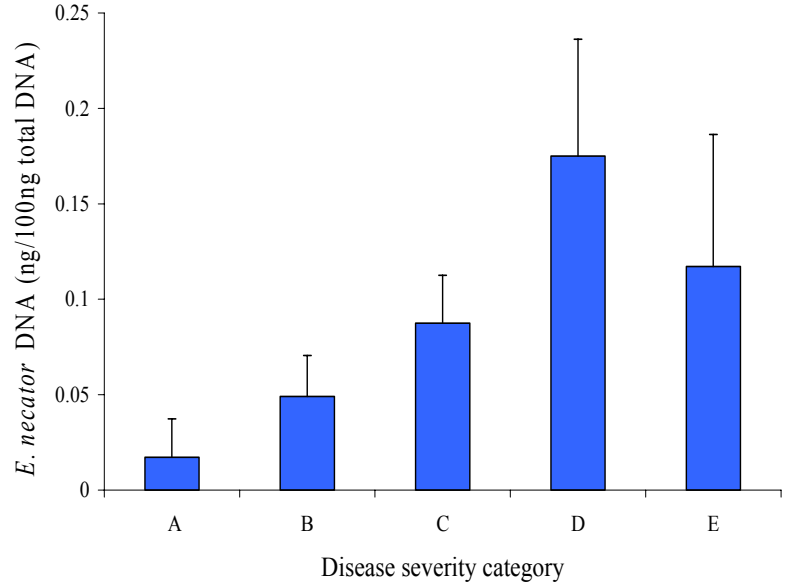
Data for vintage 2006 will now be considered in more detail. The 2006 data confirmed the assumption that visual assessment of disease is subjective. When samples from Berri Estates and Tintara were pooled there was a progressive increase in average fungal DNA concentration with increasing visual infection levels A-D, but then a decrease to level E (Figure 1 (a)). The cause of this anomaly became apparent when data from the two sites were examined separately; then the general trend of increasing fungal DNA concentration with increasing visual symptoms was apparent for both sites (Figure 1 (b), Table 4). Unfortunately, not all infection categories were available for each site. However, DNA values were higher for samples with > 30 % disease collected at Berri Estates than at Tintara vineyards. Given that *E. necator* DNA content is assumed to be constant per unit of fungal biomass, this appears to reflect differences in the interpretation of visible disease by different assessors at the independent sites. Visual assessment may be influenced by the overall amount of disease present at any one site, as less powdery mildew developed at the Tintara than at Berri Estates.

It must be noted that very few samples among the level A (no disease) group had no detectable fungal DNA, as seen with the range of values in Table 4. This indicates that the detection threshold for the DNA probe is lower than the visual detection threshold and highlights the difficulty in visually detecting small amounts of powdery mildew disease on a grape bunch. In particular, this may reflect diffuse infection, which can be detected by microscopic observation only (Gadoury et al. 2001) However, it is not known if the DNA test is able to detect DNA in residual mycelium from an earlier infection that is no longer active.

Table 4. Descriptive statistics for fungal DNA concentration (ng), visual infection category and sampling site, vintage 2006. Disease category A is uninfected; B is <5% infection; C is 5-10% infection; D is 10-30% infection; E is >30% infection. BE indicates Berri Estates, T indicates Tintara regions.

Site	Disease category	Number of samples	Mean	Std Dev	Min	Max
BE	A	4	0.01	0.01	0.00	0.02
TI	A	15	0.02	0.02	0.00	0.08
TI	B	16	0.05	0.02	0.01	0.08
BE	C	4	0.09	0.03	0.05	0.10
BE	D	4	0.18	0.06	0.10	0.25
BE	E	4	0.21	0.04	0.18	0.25
TI	E	15	0.09	0.05	0.05	0.15

(a)



(b)

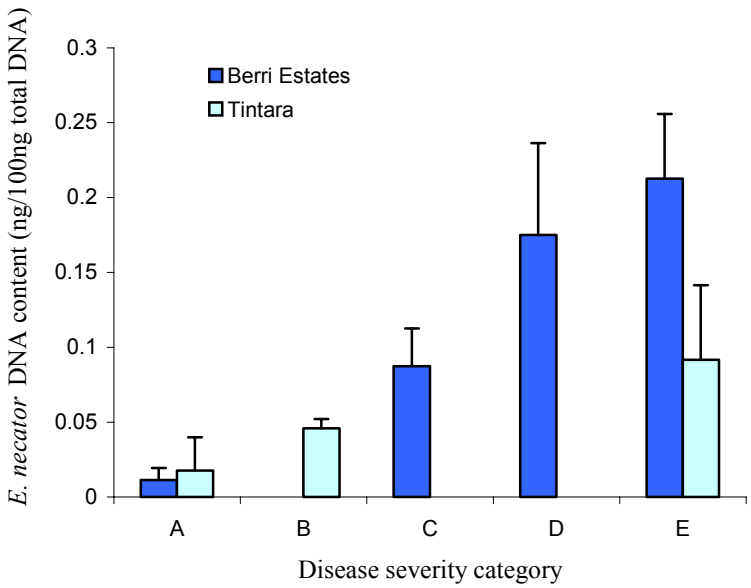


Figure 1. *E. necator* DNA content of homogenized grape samples and corresponding visual disease severity category, 2006. Category A is uninfected; B is <5% infection; C is 5-10% infection; D is 10-30% infection; E is >30% infection. Values are means, error bars represent standard deviation.
(a) *E. necator* DNA content of all grape samples combined from Berri Estates and Tintara.
(b) *E. necator* DNA content of grape samples, with data separated according to origin of sample.

NIR analysis

Raw, reflectance spectra of grape homogenates (2006) showed baseline shift over the whole wavelength range (results not shown). This is often the case with reflectance spectra as physical difference (*e.g.* particle size) can influence the spectra of samples with high turbidity (Beebe et al. 1998). This baseline shift can be removed with first derivative transformation of the spectral data, but large spectral differences could still be observed over the whole wavelength range (Appendix 5, Figure 1).

First derivative spectra were used for principal component analysis (PCA) and when the sample PCA scores are encoded with the sample information, the Berri Estates (BE) samples (in blue) form a cluster separate from the Tintara samples (in red), which show clustering related to infection level (Figure 2). Among the BE samples, one sample (BE_A13, an uninfected sample) appeared to be a spectral outlier and had very high leverage. PCA was therefore recalculated without this sample and similarly the Tintara samples clustered by infection level. This clustering was not so obvious with the BE samples – this may improve if they were analysed separately, but there were insufficient BE samples to perform a separate PCA.

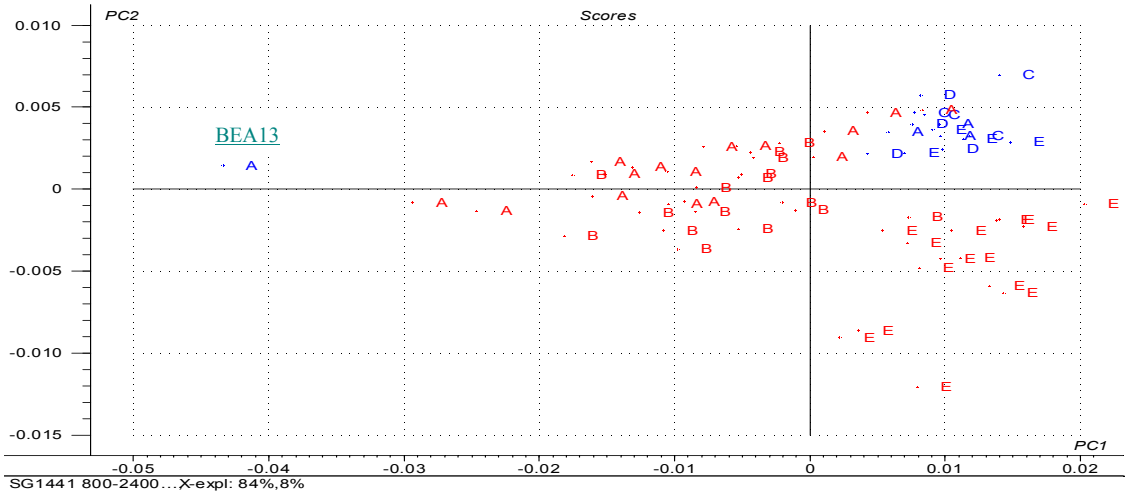


Figure 2. First 2 scores from principal component analysis of first derivative transformed reflectance spectra of homogenates of all samples, 800-2400 nm wavelength range. Berri Estates samples marked in blue, Tintara samples marked in red. Category A is uninfected; B is <5% infection; C is 5-10% infection; D is 10-30% infection; E is >30% infection

When performing discriminant analysis (DA), PCA scores have the advantage that the data are reduced (e.g. 1050 spectral data points can be reduced to 10 principal components) and the principal components have no co-linearity (Beebe et al. 1998). When all samples were used for PCA and DA, the overall prediction rate for visual infection category was 70% (Table 5). This could be due to differences in assessment between the sites or could be due to fundamental spectral differences in the chemical composition of the fruit from the two sites. Part of the problem at the lower end could be that very few of the samples were truly uninfected, as explained above.

Table 5. Classification matrix for discriminant analysis (with quadratic function) using 3 PCs from first derivative transformed spectra, 800-2400 nm, PCA outlier (BE_A13) removed. Actual classification in row header, predicted in column header. Incorrect classifications marked in red. Category A is uninfected; B is <5% infection; C is 5-10% infection; D is 10-30% infection; E is >30% infection. Data shown are with full cross-validation; in calibration mode, classification rate was 75%.

	A	B	C	D	E	% correct
A	**12**	3	2	1	0	67
B	5	**11**	0	0	0	69
C	0	0	**3**	1	0	75
D	0	0	2	**2**	0	50
E	0	1	0	3	**15**	79
Total	17	17	7	7	15	70

PCA was performed on the Tintara samples as a separate set. The samples showed clustering related to infection level and the shift with infection level was progressive (Figure 3). This distinct pattern translated to high prediction performance (100% correct for calibration, 93% correct for cross-validation) when DA was formed with the PCA scores. The incorrectly predicted samples were only with the "uninfected" and the lowest infection level (<5%) and again, this may be related to fact that there may have been very few truly uninfected samples.

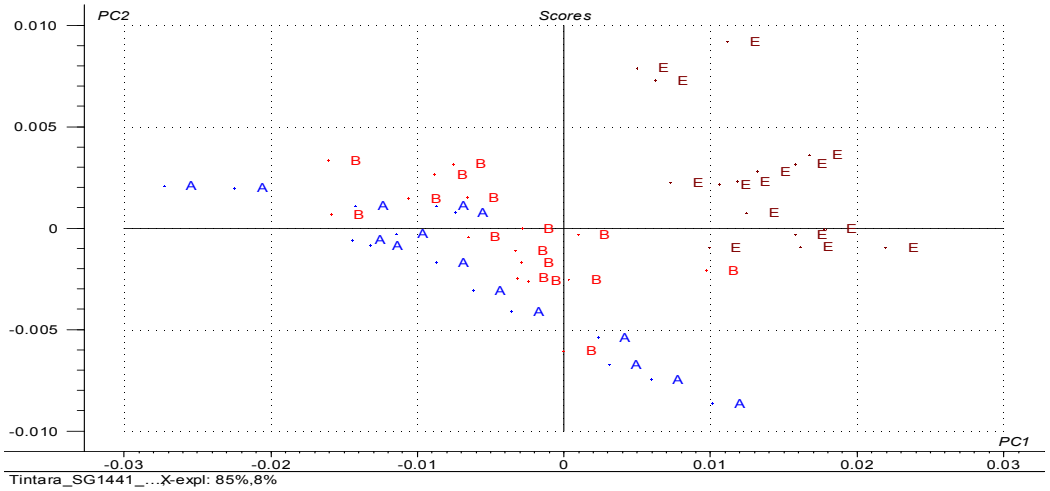


Figure 3. First 2 scores from principal component analysis of first derivative transformed reflectance spectra of homogenates, Tintara samples, 800-2400 nm wavelength range. Category A (blue) is uninfected; B (red) is <5% infection; E (brown) is >30% infection.

When analysing all samples, PCA may have been affected by bias in classification at the two sites, but this bias may be removed if the fungal DNA concentrations are used as reference information. To this end, partial least squares (PLS) regression was performed, using the spectral information to predict the DNA value. Calibration statistics using various wavelength ranges are shown in Appendix 5, Table 1. With first derivative spectra, the worst performing wavelength range (low regression coefficient and high standard error of cross-validation; SECV), was the full range (400-2500 nm). When using this range, the regression coefficients

were very noisy at wavelength >1500 nm and <600 nm (Appendix 5, Figure 2b). A restricted wavelength range spanning the end of the visible range and the start of NIR (600-1100 nm), produced a better calibration (Appendix 5, Table 1, Figure 2). The 960 nm OH stretch third overtone peak (Osborne et al. 1993) appeared to feature strongly in the NIR region (>750 nm) and if a very tight wavelength range spanning this region was used (800-1000 nm), good calibrations could be obtained (Appendix 5, Table 1).

PLS calibrations for DNA concentration were also performed with raw spectra and despite the spectral baseline shifts mentioned earlier, these calibrations performed well, particularly when using the 800-1000 nm wavelength range where the shifts are very distinct (Appendix 5, Figure 3b). The first loading (blue trace) is relatively constant over the whole range, implying that the baseline shift itself contains information related to infection level and hence DNA concentration. Figure 4 shows spectral overlays for two level A samples and two level E samples – an upward baseline shift appears to be related to infection level and is particularly distinct around the 800 nm wavelength. As discussed earlier, reflectance spectra can also give information on physical sample characteristics, such as particle size, and it is possible that fungal infection results in distinct, reproducible physical matrix alterations as well as leaving a chemical fingerprint. When the 800 nm region is examined in first derivative transformed spectra, the baseline shifts are lost, but regions related to chemical functional groups (e.g. OH region near 960 nm) show differences (Figure 5).

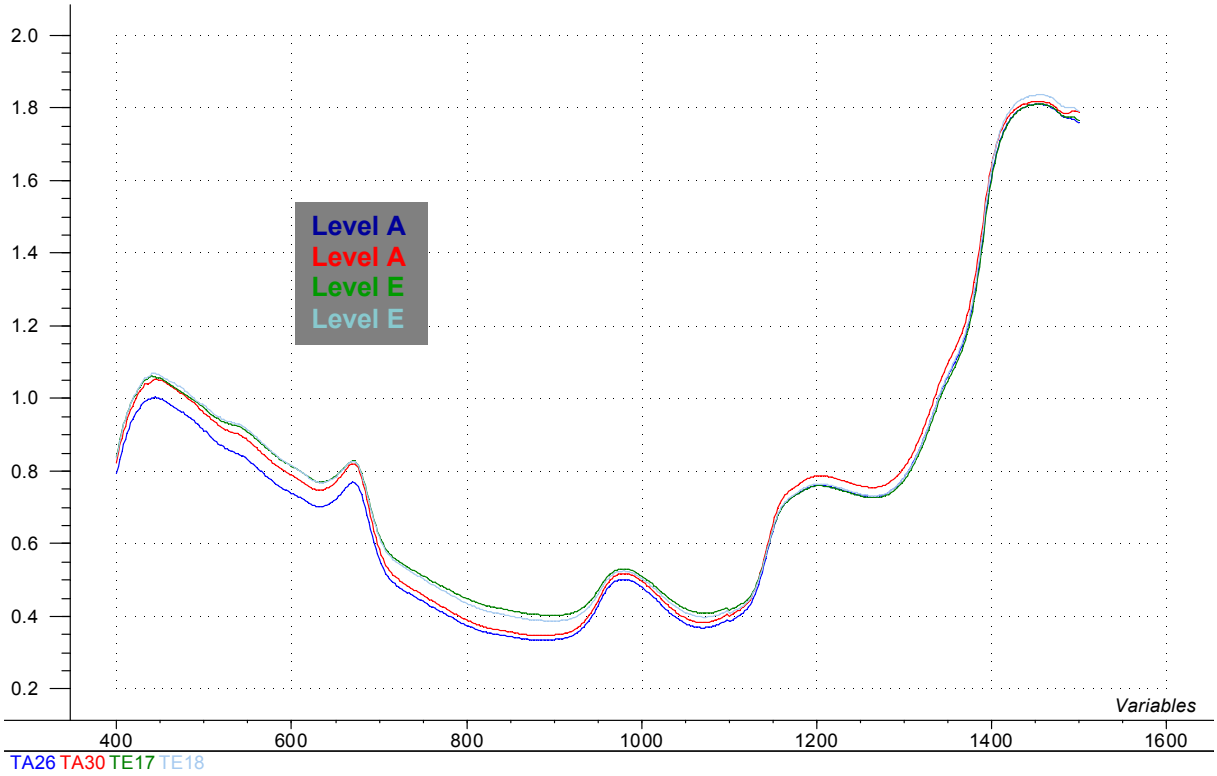


Figure 4. Raw spectra of 2 category E infected samples and 2 category A samples

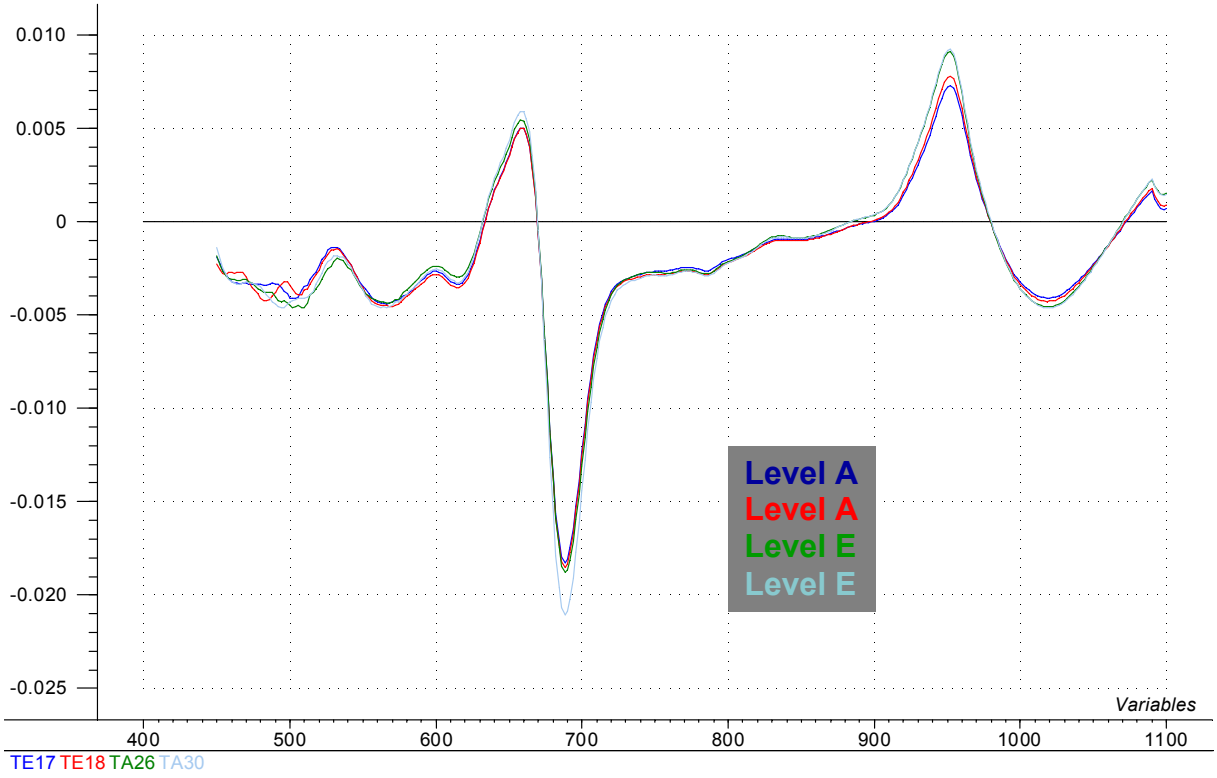


Figure 5. First derivative spectra of 2 category E infected samples and 2 category A samples.

7. Outcome/Conclusion

The relationship between disease severity, content of powdery mildew DNA and NIR spectra on samples from a range of viticultural regions was, in general, confirmed. However, this relationship was not so clear when field samples collected by industry field staff were included in the analysis and when samples were pooled from a number of sites and vintages. This could be due to inconsistencies with visual assessment. With care, training and good resources, accurate visual assessment may be possible in the vineyard, but visual assessment becomes very difficult in the winery, particularly with mechanically harvested grapes, unless disease is very severe. DNA data proved to be a more reliable reference point for NIR than did visual assessment.

NIR could provide an objective method for rapid assessment of powdery mildew contamination at the weighbridge, as has been demonstrated with previous trials and with the field samples in the present study. However, this method is correlative and requires a reference point for calibration and validation. DNA analysis would provide a reliable reference point. However, this requires that industry has access to DNA data for calibration of NIR using representative samples of grapes of the appropriate variety and with a range of powdery mildew severity to enable the successful implementation of NIR for disease assessment.

8. Recommendations

This study highlights that an objective measure of disease is required as a reference point to calibrate NIR. Visual assessment of disease appears to be inconsistent between sites and assessors. The development of readily available universal visual guides for the assessment of disease severity on grape bunches would assist in standardising disease assessment across the grape and wine industry. Such information could be communicated via field days and workshops throughout viticultural regions.

DNA analysis provided a reliable reference point for calibration of NIR. However, the slot blot assay used in this study is slow, expensive, requires use of radioactive isotopes and, therefore, is less attractive to industry than a quicker and cheaper test, such as quantitative PCR. Wine companies with NIR technology could outsource DNA-based quantification of powdery mildew for use in calibration of NIR through a diagnostic testing service. This would provide data for fungal DNA content of grape samples from a range of viticultural regions and grape varieties that could be used as a reference point for individual calibration and validation of NIR. The amount of powdery mildew DNA detected in a grape sample is not influenced by grape variety or region whereas NIR may be influenced by grape variety and composition. Therefore initial calibration of NIR instrumentation is required for each grape variety. To be of value to industry, collection of grape samples and testing of methodology in an industry setting is necessary. Accordingly, the Hardy Wine Company and the CRCV have committed additional resources to AGRF and The University of Adelaide to develop a quantitative PCR method based on the existing powdery mildew-specific clone and primers (Stummer et al. 2006). The proposed outcome of this venture is that AGRF may be in a position to provide a diagnostic service for industry. To meet this aim, additional work is needed for initial calibration of NIR for other white and red varieties in an industry setting. Application of the NIR method would provide the first practical means of quantifying powdery mildew in grape loads at the winery in real time.

9. Appendix 1: Communication

Dambergs, R.G., Stummer, B., Bevin, C., Lim, A., Cozzolino, D., Gishen, M., and Scott, E.S. (2007). Rapid analysis of powdery mildew in grapes: an industry trial. 13th Australian Wine Industry Technical Conference, Adelaide, Australia

Cozzolino, D., Dambergs, R.G., Cynkar, W.U., Janik, L., and Gishen, M. (2006). Review: Analysis of grapes and wine by near infrared spectroscopy. Journal Near Infrared Spectroscopy 14, 279-290.

Dambergs, R.G., Cozzolino, D., Cynkar, W.U., Janik, L., and Gishen, M. (2006). The determination of red grape quality parameters using the LOCAL algorithm. Journal Near Infrared Spectroscopy 14, 81-92.

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10. Appendix 2: Intellectual Property

Major outcomes might include a robust NIR-based method for detection and quantification of powdery mildew and botrytis bunch rot. Background IP relating to this matter resides with the AWRI, University of Adelaide, and CRCV. (World Intellectual Property Organisation document number WO/2007/041755)

IP would be shared between the organisations (AWRI, CRCV, UA, The Hardy Wine Company, AGRF), the funding body (GWRDC) and collaborators according to their contribution to the project. Ownership and rights to commercialise the IP arising from the research will be agreed between the parties once the funding has been secured and prior to the commencement of the research work.

Any matters likely to have bearing on IP would be discussed with stakeholders, as appropriate, prior to any public disclosure of the information.

11. Appendix 3: References

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13. Appendix 5: Data

Table A1. Calibration statistics for the prediction of fungal DNA concentration using partial least squares regression and Vis-NIR spectra of grape homogenates. SG1.4.4.1 refers to Savitsky-Golay smoothing with first derivative, 4 point gap left, right and first order polynomial function. R_cal is regression coefficient for predicted versus measured value in calibration. R_val is regression coefficient for predicted versus measured value in cross-validation. SEC is the standard error of calibration. SECV is the standard error of cross-validation.

Wavelength (nm)	Treatment	R_cal	R_val	SEC (ng/100ng)	SECV (ng/100ng)
400-2500	SG1.4.4.1	0.78	0.65	0.041	0.051
600-1400	SG1.4.4.1	0.85	0.74	0.034	0.045
600-1100	SG1.4.4.1	0.83	0.76	0.037	0.043
800-1000	SG1.4.4.1	0.76	0.72	0.043	0.045
400-2500	none	0.89	0.76	0.029	0.044
600-1400	none	0.84	0.75	0.035	0.044
600-1100	none	0.81	0.72	0.038	0.047
800-1000	none	0.92	0.79	0.025	0.041

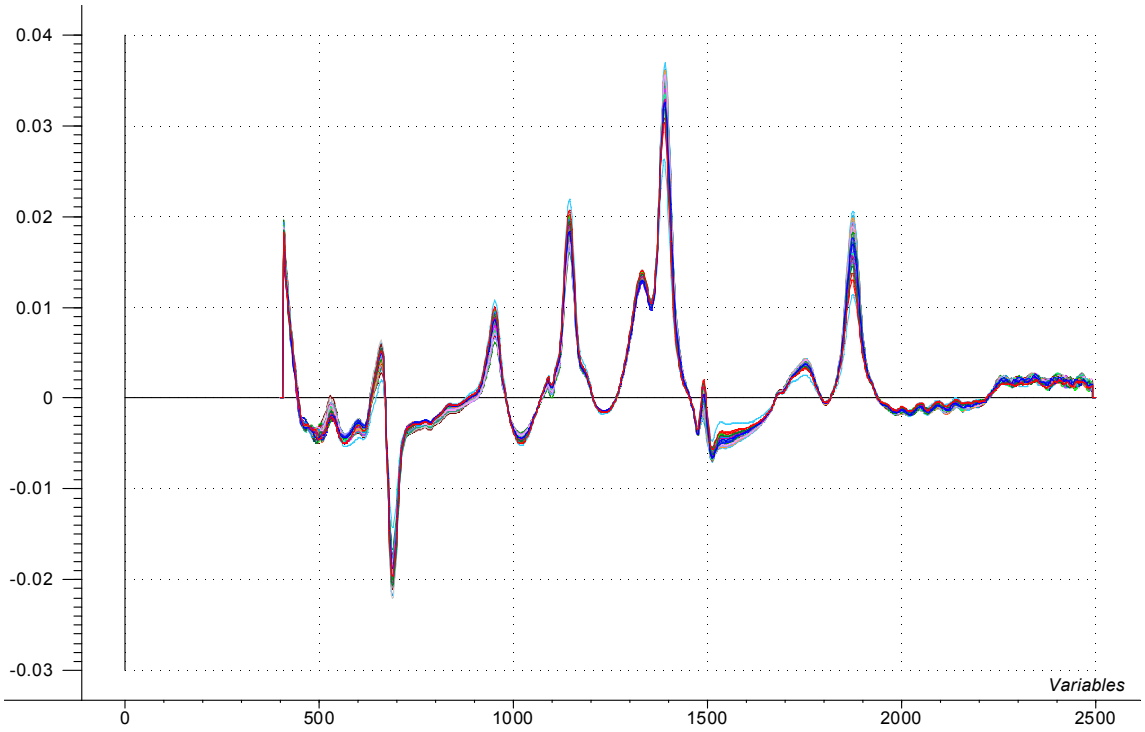


Figure A1. First derivative transformed reflectance spectra of homogenates of all samples, scanned over a 400-2500 nm wavelength range

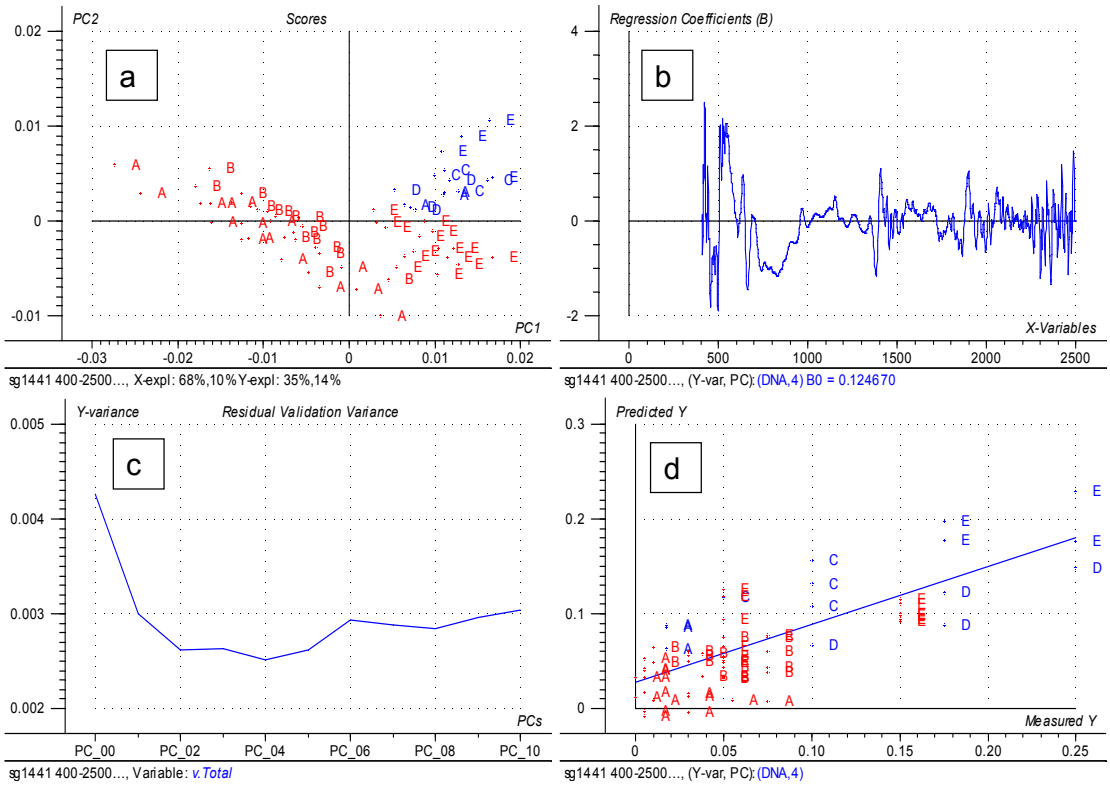


Figure A2. Overview of PLS calibration for fungal DNA using first derivative transformed spectra, 400-2500 nm, 1 high leverage sample removed (BE_A13); showing (a) first 2 PLS scores; (b) regression/beta coefficients for the calibration; (c) residual validation variance; (d) measured DNA value versus PLS predicted. Tintara samples marked in red, Berri Estates samples marked in blue. Category A is uninfected; B is <5% infection; C is 5-10% infection; D is 10-30% infection; E is >30% infection.

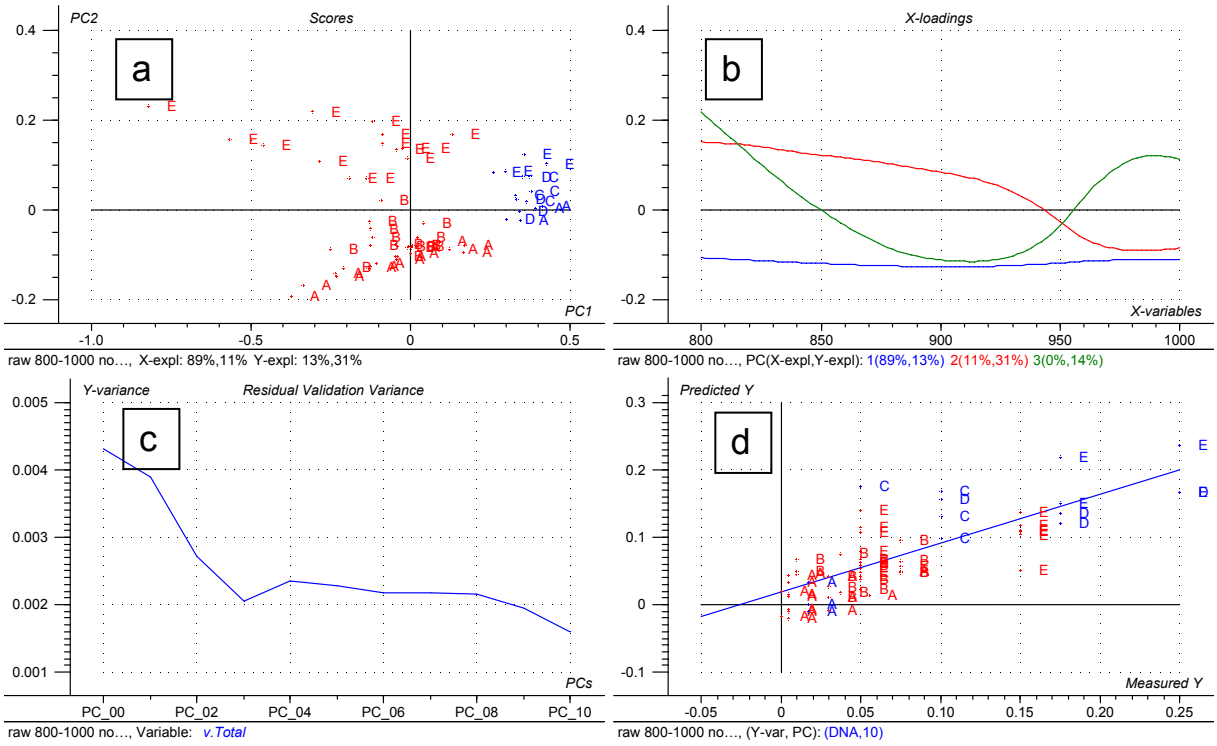


Figure A3. Overview of PLS calibration for fungal DNA using raw spectra, 800-1000 nm, 1 high leverage sample removed (BE_A13); showing (a) first 2 PLS scores; (b) loadings for the calibration; (c) residual validation variance; (d) measured DNA value versus PLS predicted. Tintara samples marked in red, Berri Estates samples marked in blue. Category A is uninfected; B is <5% infection; C is 5-10% infection; D is 10-30% infection; E is >30% infection.

13. Appendix 6: Budget reconciliation (FORM B) Statement of Receipts and Expenditure - Reconciliation Funding for 2006/07

Trust Fund :	RESEARCH TRUST FUND	FUNDING	$
Project No :		Salaries	
Grantee :		Travel	
Title of Project :		Operating	
		Capital	
		Total Funding	

EXPENDITURE										
	Salaries		Travel		Operating		Capital		Total	
	$	¢	$	¢	$	¢	$	¢	$	¢
A Uncommitted (c/f 1 July)										
B Outstanding Commitments (c/f 1 July)										
C Refunds of funding										
D Cash Received From Trust Fund										
E Approved transfers (from Form C)										
F Cash available (A+B-C+D±E)										
G Expenditure										
H Outstanding Commitments (30 June)										
I Total funds Committed (G-H)										
J Uncommitted (30 June) (F-I)										
K Other income (Paid to Trust Funds)										

Note : *Row B should be the same as Row H from the previous year and Row A the same as Row J from the previous year.*

I hereby certify that this statement of expenditure is correct.

…….	…….	…….
Signature	Printed Name	Date