## Post-bottling 'Brett' spoilage

Over the past 10 years or so, winemakers have become much more aware of the issue of 'Brett' spoilage and most have introduced measures to control this microorganism during winemaking. The decrease in the median concentration of 4-ethylphenol (the major marker compound for 'Brett') in Australian Cabernet Sauvignon wines to a level well below perception thresholds is good evidence of this (Anon 2008). However, over the same time period, the AWRI's Winemaking and Extension Services (WES) team has observed numerous wines where the concentrations of 4-ethylphenol (4-EP) increased to spoilage levels after the wines were bottled. Analysis of these wines, which were all bottled from one tank on one day, revealed different levels of 4-EP in different bottles – a phenomenon we call 'variable Brett'. That is, we define 'variable Brett' as the phenomenon whereby different bottles of the same wine contain different levels of 4-EP some time after bottling.

We have also conducted many investigations into wines where it seems there has been post-bottling 'Brett' growth, but it has not appeared to be variable (at least not at the time of analysis). These wines have contained high levels of 4-EP and either viable 'Brett' cells (isolated during microbiological analyses), or yeast cells that appear to be 'Brett' in hazes or deposits isolated from the wines. Typically in these cases, the winemakers contacted WES to investigate the development of an 'unknown sensory character/problem', or because 'the character of wine has changed', or simply to 'investigate possible Brett spoilage'. In these cases the levels of 4-EP, whilst often relatively high, have been fairly consistent and we have therefore not categorised them as 'variable Brett'.

Often in the 'variable Brett' cases, the WES team were contacted to investigate wines which had been returned from overseas (including the UK, EU, USA and Singapore) because the importers perceived them to be faulty, or because they had received complaints about the wines. Frequently in these cases, when the winemakers looked at stocks stored in Australia, the wines were assessed to be perfectly fine. Occasionally however, variability was in fact observed in Australian stock upon further investigation at the AWRI.

Given it was not known that the issue was variable 'Brett' when the investigations (which are the subject of this article) were commenced, a full set of compositional data was not necessarily obtained in each case. Depending on the initial perceptions of what the problem might have been, various tests were conducted, including sensory, microbiological and chemical analyses.

### Two typical scenarios

Whether samples of wine returned from overseas are compared to samples held locally, or whether variation is observed in samples all held in the same place, two types of scenarios typically develop:

Scenario A. A range of 4-EP levels between minimum and maximum values is observed in the samples analysed.

Scenario B. Two distinct groups of samples emerge upon analysis: those with relatively low 4-EP levels and those with relatively high 4-EP levels. There is usually slight variation in the group of samples with the high 4-EP levels.

Examples of these two scenarios are given in Figure 1.

In the case of Scenario A, it is assumed that all bottles of the wine contained viable 'Brett' cells after bottling and that the variation in 4-EP level is likely due to either variations in starting cell numbers in the bottles; variations in storage conditions; variations in the closure (discussed further below); or a combination of these factors. Observations such as the isolation of viable 'Brett' cells during microbiological analyses, variable levels of glucose plus fructose (G+F) and the presence of variable morphology yeast cells (indicative of 'Brett') in deposits isolated from samples, imply the presence of viable 'Brett' cells after bottling (see Table 1 and Figure 2).

In the case of Scenario B, we assume that not all bottles of the wine contained viable 'Brett' cells after bottling, or that if they did, they became non-viable in a portion of the bottles soon after bottling. We have observed intermittent re-fermentation issues associated with *Saccharomyces* yeast at the AWRI, so it is not surprising to observe a similar situation involving 'Brett'. This situation can arise if the wine contained very low viable cell numbers at the time of bottling, such that some bottles ended up with at least one or more cells and others ended up with no cells. Otherwise, the situation can arise through contamination of equipment at bottling, such as a portion of contaminated filler heads. In the 'Scenario B' cases we have investigated, it is possible that there might have been greater variation in 4-EP levels earlier during storage, and that the wine was tested at the AWRI after most of the 'Brett' growth, and corresponding production of 4-EP, had occurred.

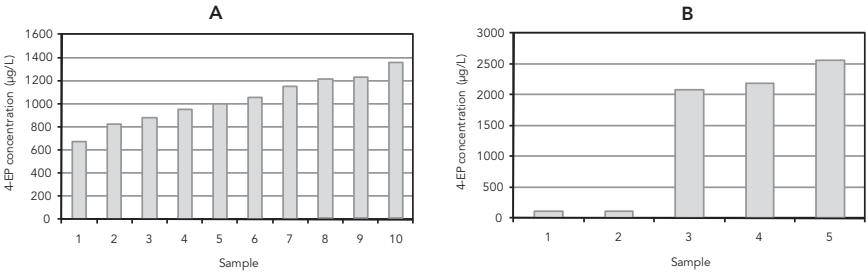


**Figure 1.** The two types of scenarios observed in 'variable Brett' cases: Scenario A, where a range of 4-EP levels is observed between minimum and maximum values; and Scenario B, where two groups of 4-EP levels are observed: a group with relatively low 4-EP values and a group with relatively high 4-EP values (there may be some variation in the group of samples with the high 4-EP levels).

**Table 1.** Examples of wines where results of analyses, including the concentration of 4-ethylphenol (4-EP level), whether or not viable *Brettanomyces* yeast were observed during microbiological analysis (Viable 'Brett' present?) and observations made during the examination of any deposit isolated (Examination of any deposit), suggested the wines were affected by 'variable Brett'.

| Wine | Comments on wine sample | 4-EP level (µg/L) | Viable 'Brett' present? | Examination of any deposit |
|---|---|---|---|---|
| Wine A | Stored in Australia | 812 | Yes | Variable morphology (ovoid to elongated) yeast (typical of 'Brett'), spherical yeast, rod-shaped bacteria and coccoid bacteria. |
| | Returned from EU | 1470 | Yes | Variable morphology (ovoid to elongated) yeast (typical of 'Brett'), spherical yeast, rod-shaped bacteria. |
| Wine B | 'Low' 4-EP sample | 245 | Yes | — |
| | 'High' 4-EP sample | 555 | Yes | — |
| Wine C | 'Low' 4-EP sample | 34 | No | — |
| | 'High' 4-EP sample | 1401 | Yes | — |
| Wine D | 'Low' 4-EP sample | 117 | No | Spherical yeast, coccoid bacteria. |
| | 'High' 4-EP sample | 2550 | Yes | Variable morphology (ovoid to elongated) yeast (typical of 'Brett'), spherical yeast, coccoid bacteria. |
| Wine E | 'Low' 4-EP sample | 674 | Yes | Variable morphology (ovoid to elongated) yeast (typical of 'Brett'), apiculate yeast, rod-shaped bacteria, coccoid bacteria. |
| | 'High' 4-EP sample | 1204 | Yes | Variable morphology (ovoid to elongated) yeast (typical of 'Brett'), coccoid bacteria |

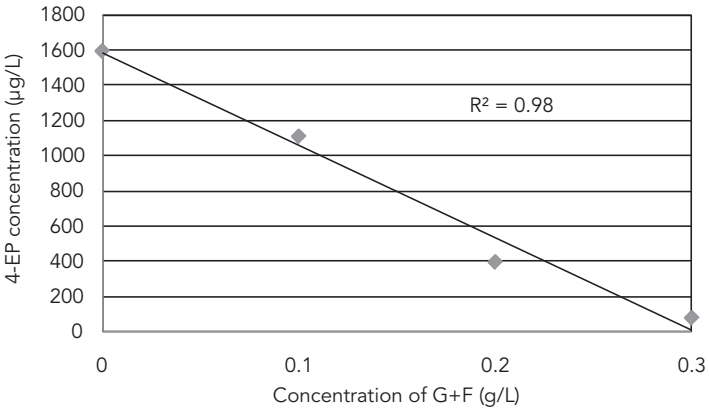


**Figure 2.** Relationship between the concentrations of 4-EP and G+F in different bottles of a 2001 Shiraz wine

## Variable 4-EP levels and oxygen ingress through closures

If we look at the 'Scenario A' case and assume that all bottles of the wine contained viable 'Brett' cells after bottling, then why do we observe variations in 4-EP? After all, we would expect the wine to be well mixed and homogeneous for bottling, which should result in each bottle having the same, or similar, 'Brett' cell numbers. It is suggested that the reason is the same as the main reason why we sometimes observe different bottles of the same wine exhibiting varying degrees of oxidation after bottling. That is, variations in the amount of oxygen entering the bottles due to variations in the oxygen transfer rate (OTR) of the closures (variations in dissolved oxygen levels between bottles might also contribute to this problem in some cases). All the cases of 'variable Brett' we have investigated have involved wines sealed with natural cork closures (which can have OTR values ranging from relatively low through to relatively high), although in the case of one particular wine, a portion was also bottled under screw cap. In this case, the portion of wine under screw cap (which have relatively low OTR values) did not exhibit any 'Brett' character and the level of 4-EP measured was relatively low.

It has been known for some time that 'Brett' growth is stimulated by oxygen. For example, Wikén et al. (1961) showed that various strains of 'Brett' undergoing alcoholic fermentation under anaerobic conditions were stimulated in the presence of molecular oxygen. More recently, Du Toit et al. (2005) showed that the addition of oxygen to wine containing a low concentration of sulfur dioxide ($SO_2$) supported the survival and growth of 'Brett'.

We have investigated many cases of variable post-bottling oxidation, the vast majority involving cork or cork-based closures. The evidence in most cases we have investigated suggests this phenomenon is related to variations in OTRs of those cork or cork-based closures, which is often exacerbated by upright storage in relatively dry conditions. Bartowsky and Henschke (2000, 2004) observed similar phenomena in various batches of red wine stored in an upright position, where variable spoilage by acetic acid bacteria was observed. The WES team has also observed this same phenomenon in red wine. Variable ingress of oxygen through the closures with corresponding enhancement of acetic acid bacteria growth has been hypothesised as an explanation for these observations (Bartowsky and Henschke 2000). Note that various authors have reported on the variation in OTR values for natural bark closures (Waters et al., 1996, Caloghiris et al. 1997, Godden et al. 2001, Jung and Zürn 2001).

It is possible the same mechanism is responsible for 'variable Brett'. That is, bottles of wine sealed with closures allowing relatively larger amounts of oxygen to permeate through (i.e. those closures with relatively high OTRs) are more likely to be the first to develop high 4-EP levels, if viable 'Brett' cells are present, due to the stimulatory effect of oxygen. It might also be that the amount of oxygen that permeates through closures with very low OTRs is insufficient to stimulate 'Brett' growth.

The fact that bottles sealed with screw caps did not appear to develop 'Brett' in the case mentioned above (where a particular wine was bottled under screw caps as well as corks), is consistent with this hypothesis. As in the case of variable post-bottling oxidation, upright storage under relatively dry conditions can increase the rate of oxygen permeation through cork and cork-based closures.

### 'Variable Brett' and volatile acidity

It has been reported that 'Brett' can produce high levels of acetic acid (Ribéreau-Gayon et al. 2000, Sponhoz 1993), however, this only occurs when 'Brett' grow under aerobic conditions (Ciani and Ferraro 1997). Whilst the oxygen that might permeate through a relatively high OTR closure can stimulate 'Brett' growth as discussed above, the amount is small relative to the amount required for oxidative metabolism. Therefore, it would not be expected that 'Brett' would cause spoilage due to acetic acid production under post-bottling conditions.

Acetic acid was measured in approximately 60% of the variable Brett cases investigated (at least one 'low 4-EP' sample and one 'high 4-EP' sample in each case, but usually multiple bottles were tested) and no variation higher than the uncertainty in the measurement for the method was observed in the results. Even when differences of approximately 2000 µg/L of 4-EP were observed between the samples with the lowest and highest levels of 4-EP, no differences in acetic acid were observed.

### How much sugar does 'Brett' need?

Whilst 'Brett' growth rate is enhanced with increasing concentrations of sugar, substantial populations may develop at sugar levels of less than 2 g/L (Fugelsang 1997). Indeed, Chatonnet et al. (1995) indicated that the utilisation of 275 mg/L (0.275 g/L) of fermentable sugars by 'Brett' was sufficient to produce 425 µg/L of 4-EP. In our study (Curtin et al. 2008) on the sensory perceptions of 'Brett' and relationship to consumer preference, we found that increasing 'Brett' character causes a significant decrease in consumer liking (104 Sydney-based red wine consumers participated in the study). We also found that group (30 member sensory panel) mean aroma perception thresholds for 4-EP ranged from 368 µg/L in a 'neutral' base wine, to 425 µg/L in a 'green' wine, and 569 µg/L in an 'oaky' wine (Curtin et al. 2008). We can conclude, therefore, that a level of 425 µg/L of 4-EP can have a negative impact on the quality of many wines.

As indicated above, full sets of compositional data were not always obtained during 'variable Brett' investigations. However, 4-EP and glucose plus fructose (G+F) results were obtained in some cases. Using the differences between samples with the lowest and highest levels of 4-EP, and differences in the corresponding levels of G+F, we can calculate the amount of 4-EP produced per unit of G+F assimilated in these cases. Table 2 shows the 4-EP and G+F data, and the calculated amount of 4-EP produced per 100 mg of G+F assimilated (µg 4-EP/100 mg G+F), for five different 'variable Brett' cases. The calculated amount of 4-EP produced per 100 mg of G+F ranged from 197 µg for

wine 5 to 502 µg for wine 2, with an average of 331 µg. These calculations show that 'Brett' could produce approximately 1000 µg/L of 4-EP from assimilation of approximately 300 mg/L (0.3 g/L) of G+F. That is, 'Brett' can produce levels of 4-EP that are well above the sensory threshold from growth on levels of residual sugar in wines that most winemakers would consider 'dry'.

It should be noted that 'Brett' are not only capable of growth and metabolism of G+F, but can also metabolise other sugars, including galactose and trehalose (Chatonnet et al. 1995, Lodder 1970) and arabinose (Ribéreau-Gayon et al. 2000), as well as other sugars such as turanose, gentiobiose and dextrin (pers. comm. C. Curtin, AWRI).

## Preventing post-bottling 'Brett' growth

A major factor in the prevention of post-bottling 'Brett' growth is the control of this yeast during the whole winemaking process. This can be achieved by implementing a range of winemaking strategies that aim to reduce the population and proliferation of 'Brett'. Areas that need to be addressed include general cleaning and sanitation, management of residual nutrients (G+F and nitrogen), sulfur dioxide and pH (these are inextricably linked), turbidity/clarification and barrel management (Coulter et al. 2003). All these winemaking aspects must be addressed concurrently as part of a holistic approach.

**Table 2.** The differences between the lowest and highest concentrations of 4-ethylphenol (4-EP) and corresponding glucose plus fructose (G+F) concentrations for five different 'variable Brett' cases. The calculated amount of 4-EP produced per 100 mg of G+F assimilated (µg 4-EP/100 mg G+F) is also shown for each wine, as well as the average calculated for the five wines.

| Wine | Comments | 4-EP level (µg/L) | Difference between 4-EP levels (µg/L) | G+F level (g/L) | Difference between G+F levels (g/L) | Amount (µg) of 4-EP produced per 100mg G+F |
|---|---|---|---|---|---|---|
| 1 | Lowest 4-EP sample | 87 | 1963 | 0.7 | 0.6 | 327 |
| | Highest 4-EP sample | 2050 | | 0.1 | | |
| 2 | Average of group of low 4-EP samples | 84 | 1506 | 0.3 | 0.3 | 502 |
| | Highest 4-EP sample | 1590 | | nd | | |
| 3 | Average low level | 455 | 944 | 0.9 | 0.3 | 315 |
| | Average high level | 1399 | | 0.6 | | |
| 4 | Screwcap sample | 34 | 1253 | 0.5 | 0.4 | 313 |
| | Average of affected samples | 1287 | | 0.1 | | |
| 5 | Average of low 4-EP samples | 792 | 393 | 0.1 | 0.2 | 197 |
| | Average of high 4-EP samples | 1185 | | 0.3 | | |
| | **Average level of 4-EP (µg/L) produced per 100 mg of G+F =** | | | | | **331** |

Even if your 'Brett' control strategies seem to have worked and your wine isn't showing any signs of being affected by 'Brett', microbiological testing should be conducted in conjunction with chemical analysis before bottling. If chemical and microbiological analyses reveal the wine is at risk of post-bottling 'Brett' growth (i.e. viable 'Brett' cells and residual sugar present), then the winemaker will need to stabilise the wine by either: i) subjecting the wine to sterile (membrane) filtration; or ii) using antimicrobial chemical additives to inhibit or kill the 'Brett' cells. Note that filtration is the better and most preferred approach, whether for controlling 'Brett' or any other unwanted microbial activity, and the use of chemicals should be only be considered as a 'backup' to filtration.

Sterile filtration should be performed using 0.45 μm pore size membranes, followed by sterile bottling – it is essential that all equipment downstream from (and including) the filter assembly has been sterilised with either hot water or steam. If control strategies have not eliminated 'Brett' during the winemaking and the pH and level of $SO_2$ in the wine at the time of bottling are inadequate to kill the 'Brett' cells, then it is possible that other microorganisms such as bacteria might also be present in the wine. Whilst membranes of 1.0 μm pore size might remove most yeast cells, 0.45 μm membranes are required to eliminate all yeast and bacteria.

Apart from $SO_2$, the use of antimicrobial chemical additives to control wine microorganisms is generally limited to sorbic acid or dimethyl dicarbonate (DMDC). Although sorbic acid is generally effective in controlling *Saccharomyces* sp. yeast, it has limited activity towards 'Brett' and other spoilage yeasts. Sorbic acid also has little inhibitory activity towards lactic acid bacteria (LAB) and acetic acid bacteria (Fugelsang 1997). In fact, some LAB are able to utilise sorbic acid which results in the formation of the volatile compound 2-ethoxyhexa-3,5-diene, which has an intense geranium-like aroma (Zoecklein et al. 1995). This effectively rules out the use of sorbic acid in red wine unless the wine is 0.45 μm membrane filtered and sterile bottled, and the absence of LAB is confirmed post-bottling with microbiological analysis.

Whilst DMDC is effective at controlling low contamination rates of yeasts such as 'Brett' (Renouf et al. 2008), it is not very effective against LAB and acetic acid bacteria at levels below the maximum (200 mg/L) level allowed in wine (Costa et al. 2008). As with most antimicrobial chemical additives, the effectiveness of DMDC is dependent on the number of cells present, with higher cell counts leading to a decrease in effectiveness. As mentioned above, it is likely that the microbial load in a finished wine will be comprised of both yeast and bacteria. Given that DMDC is ineffective against wine bacteria, it would be preferable to sterile membrane filter a microbiologically unstable wine rather than adding DMDC. Note that we believe a properly performed sterile filtration has no detectable negative sensory effect on the product. Even if there is a slight negative effect, the benefits of filtering a microbiologically unstable wine far outweigh any negatives.

## Conclusion

Post-bottling production of 4-EP can occur in wines that contain viable 'Brett' cells. Even very small levels of residual sugar, levels that most winemakers would consider 'dry', can be utilised by 'Brett' to produce levels of 4-EP that are well above the sensory threshold. If microbiological analysis reveals that a wine contains viable 'Brett' cells, then the wine should be subjected to sterile, 0.45 μm membrane filtration before bottling. If the wine is sterile filtered and the bottling equipment downstream from the filtration unit is sterile, then it is extremely unlikely there will be any post-bottling 'Brett' growth, or growth of any other microorganisms.


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