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Current Research Efforts Regarding 4-Ethylphenol and 4-Ethylguaiacol in Wines from the Okanagan Valley Region of British Columbia, Canada

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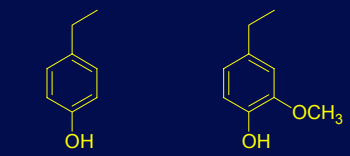
Introduction to 4-Ethylphenol, 4-Ethylguaiacol, and the *Brettanomyces/Dekkera* Connection

- *Brettanomyces* ("Brett") is a wild yeast implicated in the spoilage of red wine and has long been associated almost exclusively with European wines, but in recent years is considered to occur in wines from the New World
- Two compounds that are widely considered to be primarily responsible for the 'Brett' odour are 4-ethylphenol and 4-ethylguaiacol:

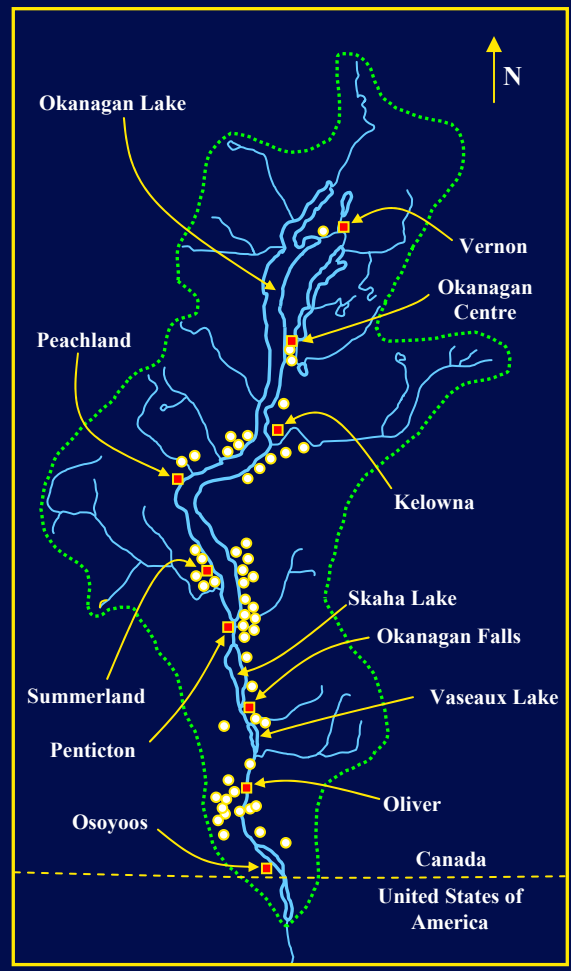


4-Ethylphenol 4-Ethylguaiacol

- *Brettanomyces* (the asexual, nonsporulating form) and *Dekkera* (its sexual, sporulating form) are ubiquitous in the vineyard and winery, and are likely to be present in the water, the soil, the grapes and must, and throughout the winery – and their presence can be monitored, but not controlled (Arvik and Henick-Kling, 2002)
- Once this yeast is established in a winery, it is difficult to eliminate → spoilage of wine by *Brettanomyces* can be devastating and wineries have had to shut down to remove this contaminant
- Maintaining appropriate sulfur dioxide (SO_2) levels, lower temperatures (generally <20°C), filtration, excluding oxygen ingress into the wine during topping up of barrels (and keeping the barrels topped up), and general winery hygiene have all been noted as methods to keep "Brett" problems minimized (Arvik and Henick-Kling, 2002)
- However, infected barrels cannot effectively be sterilized due to their large internal surface areas and porosity, whether it be by washing with sulfited water, shaving and firing, or ozone treatment (Arvik and Henick-Kling, 2002 and their citation of Kunkee, 2001; Pollnitz *et al.*, 2000a), with *Brettanomyces* found as deep as 8 mm into the oak wood (Malfeito-Ferreira *et al.*, 2004)
- For these reasons, we recently (in May 2006) began a comprehensive two-year study based in the Okanagan Valley wine region of British Columbia, Canada, that is targeted at better understanding the levels, distributions, fate, and factors governing the production of the two most well-known *Brettanomyces* metabolites: 4-ethylphenol and 4-ethylguaiacol

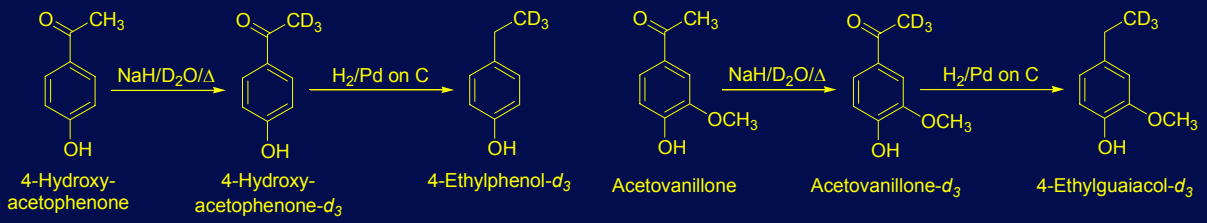
Overview of the Okanagan Valley Wine Industry

- The Okanagan Valley is located in south-central British Columbia, Canada, approximately 300 kilometers from the Pacific Ocean, is long and narrow, and runs northward for 160 kilometers from the US border at 49 to 50°N latitude
- Within the valley, there are significant climatic differences from north to south, with the following five designated agricultural sub-regions (BCWI, 2006a):
  1. **Kelowna:** 1200 degree days; heavier soils with sandy loam, clay and limestone; common varietals: Pinot Noir, Pinot Gris, Pinot Blanc, Riesling, and Chardonnay.
  2. **Penticton/Naramata:** 1320 degree days; long frost-free autumn due to lake proximity and sloping aspect; common varietals: Pinot Noir, Pinot Gris, Pinot Blanc, Chardonnay, and Merlot.
  3. **Okanagan Falls:** 1410 degree days; diverse soils and aspects, with some vineyards on terraced slopes; common varietals: Riesling, Gewurztraminer, and Pinot Noir.
  4. **Oliver/Golden Mile:** 1480 degree days; well-drained gravel, clay and sandy soils; common varietals: Merlot, Chardonnay, and Gewurztraminer.
  5. **Black Sage/Osoyoos:** 1490 degree days; soils are very deep sand; common varietals: Bordeaux varieties, Chardonnay, and Syrah
- Grape and wine production has increased steadily from 4850 tons in 1992 to a high of 16 900 tons in 2003, with the value of British Columbia VQA (Vintners Quality Alliance) wines sold in the province also increasing from CDN$6.9 million in 1991/1992 (BCWI, 2005) to CDN$131 million in 2005 (BCWI, 2006b)
- The top five red varieties produced in British Columbia during 2005 were as follows: Merlot, 2784 tons; Cabernet Sauvignon, 1054 tons; Pinot Noir, 963 tons; Cabernet Franc, 594 tons; and Syrah, 408 tons (BCWI, 2006c).



Wine Sampling and Analysis

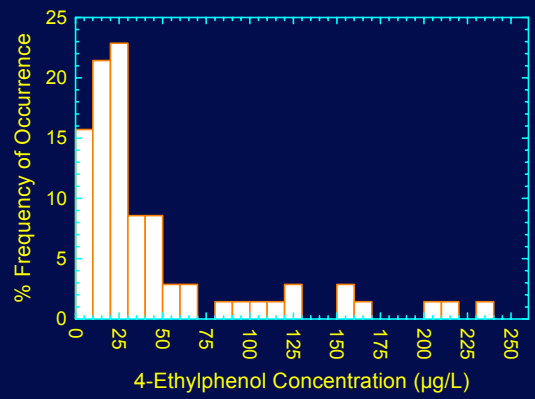
- 71 red wine samples from five major varieties (Merlot, Cabernet Sauvignon, Syrah, Pinot Noir, and Cabernet Franc) were collected using sterile glassware from ~220 L oak barrels at 7 estate- through large-scale wineries in the Okanagan Valley
- 10 commercially available un-oaked bottled Riesling wines from Canada and Europe (vintage years 2002 through 2004) were also analyzed
- Sample volumes ranged from 50 mL to 250 mL, were collected using glass pipettes, stored at 4°C without headspace in pre-cleaned (Alconox detergent, following by sequential rinses with tap water, deionized water, dichloromethane, toluene, and hexanes) and pre-baked (300°C for 12 hours) amber glass jars until analysis
- At the time of sampling, dissolved oxygen and temperature were measured at half-depth in the barrel using a commercial meter. Other variables (e.g., barrel type and details, cellar temperature and humidity, barrel hygiene and history, etc.) were obtained via direct observation and/or personal communications with individual winemakers.
- The corresponding stable isotope derivatives (4-ethylphenol-d_3 and 4-ethylguaiacol-d_3) of the target analytes were synthesized using base-assisted deuterium exchange on the terminal methyl group from 4-hydroxyacetophenone and acetovanillone, respectively, followed by palladium-catalyzed hydrogenation of the carbonyl group:

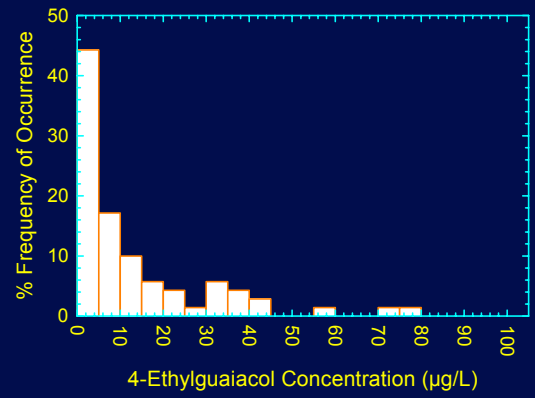


- Wines were analyzed by spiking a known quantity (5 mL) of sample with the isotopically labeled internal standards, saturating the solution with salt (NaCl) to obtain a consistent ionic strength between samples, extracting with diethyl ether (2 mL), and centrifugation to break the partial emulsion and maximize analyte recoveries
- The resulting isolated organic extracts were dried under ambient air for 12 h to evaporate the solvent, reconstituted in 50 µL of diethyl ether, and 5 µL was injected into a gas-chromatograph/mass-spectrometer (GC-MS; Saturn 3800/2000) running in selected-ion-monitoring (SIM) mode

Results to Date and Comparison with Levels from Other Worldwide Winemaking Regions

- Concentrations of both 4-ethylphenol and 4-ethylguaiacol in randomly selected barrelled red wines analyzed in 70 samples from 7 commercial wineries in the Okanagan Valley were all below aroma thresholds (~500 µg/L for 4-ethylphenol and ~50 µg/L for 4-ethylguaiacol)
- About 60% of the samples contained 4-ethylphenol concentrations below 30 µg/L (with a method detection limit of 10 µg/L), with all samples below 235 µg/L
- Similarly, about 60% of the samples contained <5 µg/L 4-ethylguaiacol (with a method detection limit of 1 µg/L), and all samples had less than 80 µg/L
- In comparison with concentrations reported from other worldwide winemaking regions, the levels of 4-ethylphenol and 4-ethylguaiacol we found in the Okanagan barrelled reds are quite low:
  - Chatonnet *et al.* (1992) analyzed 137 red and white wines from France, and found that red wines contained an average of 440 µg/L 4-ethylphenol (range: 1-6047 µg/L), compared to white wines having an average of 3 µg/L 4-ethylphenol (range: 0-28 µg/L) → some French wineries contained up to half of their vintages with 4-ethylphenol sourced "Brett" organoleptic defects
  - In a survey of barrelled (1998 vintage) and bottled (1986-1996 vintages) red wines from Australia, Pollnitz and co-workers reported barrelled 4-ethylphenol ranges of 385-680 µg/L and bottled ranges of 2-2660 µg/L (mean=795 µg/L) (Pollnitz *et al.*, 2000a, 2000b) → corresponding ranges of 4-ethylguaiacol were 28-45 µg/L in barrelled reds and 1-437 µg/L (mean=99 µg/L) in bottled reds.
  - In a subsequent survey of Cabernet Sauvignon and Cabernet Sauvignon-Merlot wines from the vintages 1996-2002 in Australia, Henschke *et al.* (2004) found that mean 4-ethylphenol concentrations in the years between 1996-2000 were not different (range from 864 to 1164 µg/L), but that concentrations decreased to an average of 490 µg/L for the following 2001 and 2002 vintages → Australia is observing a decreasing incidence of red wines with 4-ethylphenol concentrations above 800 µg/L after 2001 (Coulter *et al.*, 2003; Hayasaka *et al.*, 2005).





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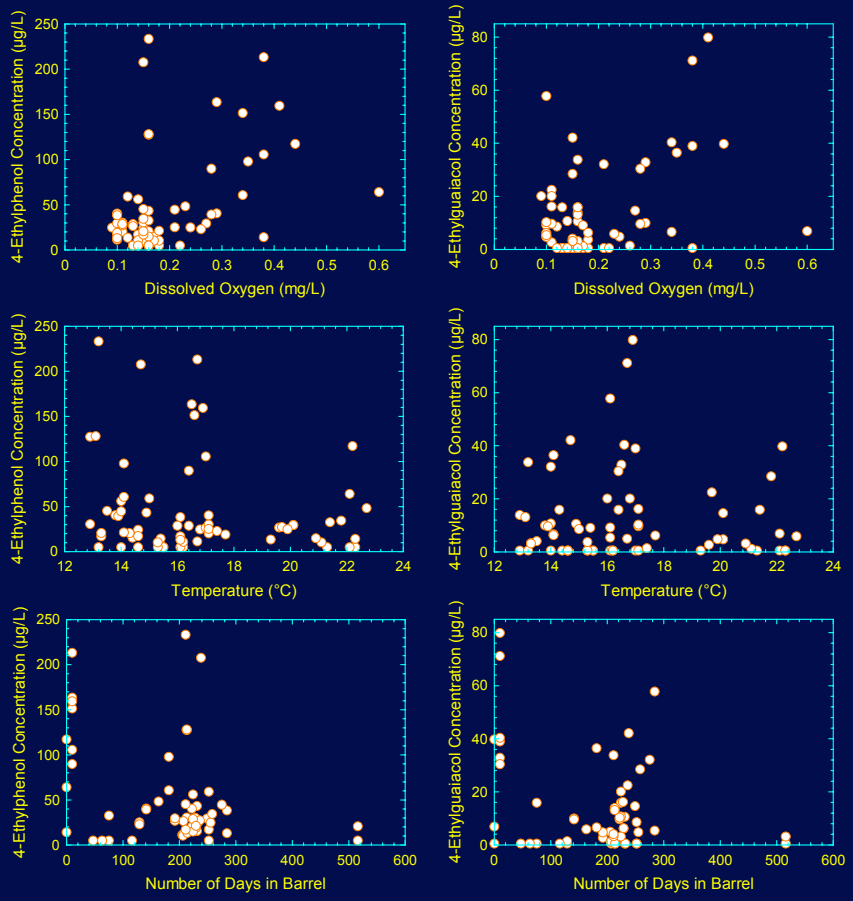
Relationships Between 4-Ethyphenol/ 4-Ethylguaiacol Levels and Other Wine Variables

- To date, we have found no significant relationships ($p<0.05$) between 4-ethylphenol and 4-ethylguaiacol concentrations and the following parameters: levels of dissolved oxygen in the barrel, temperature of the barrelled wine, or the number of days the wine had been in the barrel prior to our sampling.
- As well, we found no significant differences in average 4-ethylphenol or 4-ethylguaiacol concentrations between grape varieties, barrel oak type, barrel toasting level, barrel hygiene procedure, barrel age, cooper of the barrel, number of rackings prior to sampling, or cellar humidity (data not shown)
- In general, what we (and other previous research groups) observe between these variables is that the large variability and typically low analyte concentrations reported result in no statistical differentiation
- Furthermore, no significant differences in average 4-ethylphenol or 4-ethylguaiacol concentrations were found between the individual wineries participating in our study



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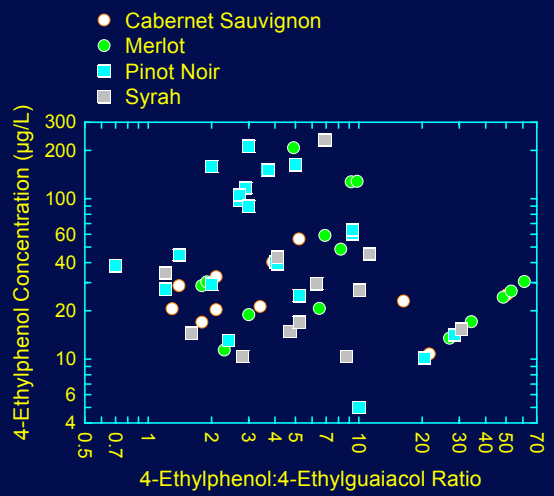
4-Ethylphenol:4-Ethylguaiacol Ratios as Potential Signatures of "Brett" Contamination

- Attention has also been paid to the observed ratios of 4-ethylphenol to 4-ethylguaiacol concentrations in the literature, and the potential diagnostic role of this ratio in separating background levels from 'Brett-derived' sources
- As with other investigators (Chatonnet *et al.*, 1992; Polnitz *et al.*, 2000a, 2000b), we found an average ratio of 10:1 among all samples pooled together, although our pooled sample median was 5.2, and the range was large (from 0.7 to 61).
  - Polnitz *et al.* (2000a, 2000b) reported a decrease in the mean ratio among varieties in the following order: Cabernet Sauvignon, 10:1; Syrah, 9:1; Merlot, 8:1; and Pinot Noir, 3.5:1 (only the difference in ratios between Syrah and Pinot Noir was noted as statistically significant)
  - We found the following order of mean ratios between variety: Merlot, 16:1; Cabernet Sauvignon, 10:1; Syrah/Shiraz, 8:1; and Pinot Noir, 6:1 (only the difference in ratios between Merlot and Pinot Noir was statistically significant)
- Given the variability in ratios (and the underlying analyte concentrations) in our study and from other research groups, our ratios are considered within the range previously reported in the literature, and further suggest that the ratio of 4-ethylphenol to 4-ethylguaiacol concentrations cannot be used as a reliable discriminator of Brettanomyces-sourced analytes from their background levels

Table: Summary statistics for 4-ethylphenol and 4-ethylguaiacol concentration ratios in Okanagan barrelled red wines by variety.

Variety	Number of Samples	Mean	Median	Min	Max	Std Dev
All	70	10.1	5.2	0.7	61	13
Merlot	21	16.1	10.0	1.8	61	18
Cabernet Sauvignon	13	10.0	3.9	1.3	50	14
Pinot Noir	22	6.1	3.4	0.7	28	7
Syrah	12	7.8	5.8	1.2	31	8



Conclusions and Recommendations for Future Work

- The 4-ethylphenol (~40-50 µg/L) and 4-ethylguaiacol (~10-15 µg/L) concentrations measured to date in barrelled Okanagan red wines are about 10-fold lower than concentrations reported in similar wines from Australia and France
- Based on a comprehensive literature review and critical examination of potential 4-ethylphenol and 4-ethylguaiacol sources in the winemaking process, we estimate that "background" levels of these two analytes are in the range of <50-100 µg/L and <10-20 µg/L, respectively
  - These conclusions are supported by our analyses of a barrelled Okanagan Chardonnay (28 µg/L 4-ethylphenol and 6 µg/L 4-ethylguaiacol) and 10 commercially available un-oaked bottled Riesling wines from Canada and Europe (vintage years 2002 through 2004) which contained between <10 to 25 µg/L of 4-ethylphenol (mean=median=17 µg/L) and all but one Riesling sample had <1 µg/L of 4-ethylguaiacol (with the remaining German Riesling having a 4-ethylguaiacol level at 1.4 µg/L)

Dr. Nigel Eggers (left) and Dr. Sierra Rayne (right) at one of the participating wineries

- Future work is being concentrated on the following key aspects of our study:
  - Continued monthly monitoring of the 2005 Merlot, Cabernet Sauvignon, Pinot Noir, Syrah, and Cabernet Franc red wine vintages at participating wineries, thereby expanding the available survey dataset for this vintage and providing temporal data within individual barrels → new temporal studies will begin with the 2006 and 2007 vintages once available
  - Performing novel laboratory experiments to develop a better understanding of the relative contributions from "free" (i.e., sensorily active) and "bound" (sensorily inactive) forms of 4-ethylphenol and 4-ethylguaiacol, such as glycosides and covalent/non-covalent associations with anthocyanins and other polyphenols in wines
  - Investigating spatial patterns of analyte distributions within a barrel, such as partitioning into/onto the oak wood and yeast lees

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