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REVIEWS

Mousy Off-Flavor: A Review

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Although mousy off-flavor occurs infrequently in wine, it can be economically disastrous to the wine producer as, at worst, it can render the wine unpalatable or, at best, decrease the quality of the wine resulting in a lower sale price. Wines infected with either lactic acid bacteria (LAB) (particularly heterofermentative strains) or *Dekkera*/*Brettanomyces* yeast can potentially produce mousy off-flavor. There are three known compounds that cause mousy off-flavor: 2-ethyltetrahydropyridine, 2-acetyltetrahydopyridine, and 2-acetylpyrroline. *Dekkera*/*Brettanomyces* have been shown to be capable of producing at least two of these compounds, whereas LAB are capable of producing all three. The reason as to why mousy off-flavor forms in some wines and not in others is still not fully understood. The issue is further complicated by the fact that the compounds that have thus far been identified as necessary for off-flavor formation are all potentially available in wine (e.g., ethanol, L-lysine, L-ornithine, and metal ions). For these reasons, the microbe's metabolism probably plays a key role in mousy off-flavor formation. In the case of *Dekkera*/*Brettanomyces*-induced mousy off-flavor, it appears that oxygen may play a key role. Thus, a wine infected with *Dekkera*/*Brettanomyces* in the absence of oxygen may not become mousy unless exposed to oxygen via a processing or handling procedure.

Keywords: Mousy off-flavor; wine; *Dekkera/Brettanomyces*; lactic acid bacteria; 2-ethyltetrahydropyridine; 2-acetyltetrahydropyridine; 2-acetylpyrroline

INTRODUCTION

Mousy off-flavor is a sporadic yet potentially disastrous problem, which has been estimated to incur a significant annual loss to the beverage industry. Mousy off-flavor, in one of the earliest notations, has been described as a "peculiarly disagreeable flavor in wine, which is closely resembling to the smell of a residence of mice." Mousy off-flavor is a consistent underlying problem for the wine industry (*1*, *2*). The reported incidences of mousy off-flavor are increasing. Whether this is an actual increase in the number of cases or an increase in discrimination of the off-flavor due to better education is unknown. Currently, there is no method available to remove mousy off-flavor from wine, and once infected, the wine becomes unpalatable.

The earliest reports of mousy off-flavor incidence in wine were made in the late 19th century (*3*, *4*). Since then, anecdotal reports have become more numerous and widespread. Mousy off-flavor occurrence in wine has been reported in both old and new world wine-producing countries, such as France (*3*, *5*, *6*), the United States (*7*), South Africa (*8*), and Australia (*9*−*12*). This is the first attempt to review the literature related to mousy off-flavor.

HISTORY

For over 100 years, many theories as to the origins of mousy off-flavor have been put forward and sequentially dismissed. The cause of mousy off-flavor was initially suggested to be bacteria-produced acetamide (*3*). However, it was reported in 1889 that pure acetamide was odorless (*13*), and it was later found that the mousy odor associated with it was due to the impurity, 2,4,6-trimethyl-1,3,5-triazine, neither of which have been isolated from wine (*14*). Although acetamide has been found to be odorless in its pure form, it has been continually argued in the literature as the cause of mousy off-flavor to the present day (*14*−*16*).

Mousy off-flavor production has also been linked with yeast autolysis, where it was believed to occur from long contact with lees in a warm environment (*17*). This author also reported that a higher than normal concentration of volatile acidity was produced under anaerobic conditions in conjunction with mousy off-flavor, rejecting the idea that acetic acid bacteria (AAB) were involved in the process. Contrary to this, it was later found that 27 strains of *Acetobacter aceti* were capable of producing

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mousy off-flavor in a grape juice medium (*7*, *18*) as well as in fortified wine (*19*). Furthermore, strains of AAB can grow anaerobically, using phenolic compounds as terminal electron acceptors (*20*). Thus, AAB should not be discounted as potential producers of mousy off-flavor.

Other studies have focused on a potential link between the redox potential (rH) of wine and the formation of mousy off-flavor. It was found that mousy off-flavor could be chemically induced in wine by oxidative processes, which caused the wine to have a high rH (*21*, *22*), and that this mousy off-flavor could be induced or removed by increasing or decreasing the rH (*22*).

Certain physiochemical treatments have been observed to instigate the formation of mousy off-flavor. Ultrasonic treatment of wine enhanced mousy off-flavor in both the presence and the absence of a copper membrane (*23*, *24*), as did γ-radiation (*25*, *26*). Another study showed that the physiochemical conditions necessary for mousy off-flavor formation were high rH values (*20*−*26*) and pH, exposure to atmospheric oxygen, the presence of sufficient "active" iron, and possibly low concentrations of tannins, pigments, and sulfur dioxide (*27*, *28*).

Other than the proposition that acetamide was responsible for mousy off-flavor formation in wines, few suggestions have been put forward as to the nature and origin of the causative compound(s). This is mainly due to the lack of sufficiently sensitive analytical procedures and equipment available at the time. Villforth (*29*) (cited in *28*) found that the causative compound was steam volatile at atmospheric pressure, and experiments showed that the compound was not an ester. He proposed that the compound responsible for mousy off-flavor was a polymer of acetaldehyde or formaldehyde, as the off-flavor became weaker with the removal of aldehyde from solution. Similar conclusions were drawn from two other studies after noting the ability of the causative compound to bind with sulfur dioxide (*22*, *23*). Unguryan and co-workers (*27*) suggested that the unknown substance was a chemically unstable nitrogen-containing substance ($R-NH_2$), which occurred at extreme rH values.

On the basis of the physicochemical nature of the mousy off-flavor compound(s), a number of methods of removal were found to be successful, including cation exchange resins to remove the suspected $R-NH_2$ compounds (*23*), strong oxidative procedures utilizing ozone treatments (*30*), and fortifying the wines (*31*).

Tucknott (*28*) conducted a major study on mousy off-flavor, examining the variation in tasters' ability to be able to detect the off-flavor, the microorganisms involved in its formation, and the chemical nature of the compound(s) thought to be involved. Different strains of yeast and bacteria, which had been previously isolated from mousy ciders, were tested for their ability to produce mousy off-flavor in a modified apple juice medium. Only one yeast, *Dekkera anomala* (previously classified as *Brettanomyces anomalus*), was found to produce the off-flavor. Mousy off-flavor was also found to occur if lactobacilli were cocultured with fermenting yeast (*Saccharomyces* spp.) in the presence of ethanol and L-lysine.

Through the use of gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS) analysis, Tucknott proposed that the compound responsible for mousy off-flavor was 2-ethyl-Δ^1-piperideine(2-ethyltetrahydropyridine). However, because of insufficient quantities of the compound being isolated, he was unable to confirm this result by infrared and nuclear magnetic resonance studies. Compounds with a mousy odor, other than 2-ethyltetrahydropyridine (ETHP), were also detected in the GC spectrum; however, the compounds responsible were not of sufficient concentration for identification, suggesting that more than one compound may be responsible. Tucknott (*28*) found that mousy off-flavor could not be induced by physicochemical means in unfermented raw materials, thus highlighting the importance of microorganisms.

Reports of mousy off-flavor occurrence in the literature are sparse, and there are numerous reasons for this. The first of these is that it has only been relatively recently that there has been an increase in awareness of the condition (*32*, *33*). Also, mousy off-flavor usually occurs in combination with other wine faults, such as oxidation and volatile acidity, making it harder to identify. Wine producers appear hesitant to admit to having a problem with mousy off-flavor occurrence, as they fear that it will reflect badly on the winery. Possibly a reason for sparse citation, however, is due to individuals' varying sensitivity to the off-flavor itself, which is discussed in detail below.

SENSORY ASPECTS

Mousy off-flavor is usually perceived in a delayed fashion on the palate, generally after the wine has been swallowed or expectorated. Once detected, it can persist for more than 10 minutes (*32*, *34*). The off-flavor, however, cannot be perceived by sniffing the wine as the compounds responsible for mousy off-flavor are not sufficiently volatile at wine pH (*32*, *34*), as described in detail below.

An individual's ability to discriminate mousy off-flavor in wine at low concentrations is genetically predisposed, which results in variation in perception (*28*, *32*, *35*). There is a correlation between an individual's mouth saliva and tongue surface pH and their ability to detect the off-flavor, with some individuals extremely sensitive to the taste while others appear totally anosmic. For this reason, we speculate that, for mousy off-flavor to be detected, an increase in pH is required to convert the causative compound into its more basic form. The correlation between salivary pH and ease of detection may reflect the lack of mousy off-flavor reports in the past.

Techniques have been developed to detect mousy off-flavor in wine without actually tasting the wine itself due to its unpleasant taste. The first, the "palm and sniff" method, involves rubbing the wine on the back of the hand and sniffing close to the skin (*32*). The other method is an alkaline strip method (*11*, *36*, *37*), where an alkaline strip is dipped into the wine and then sniffed. Both methods are quick and effective in allowing the detection of mousy off-flavor in affected wine samples. Oxidation may also be used as a method of mousy taint detection (*32*), as it has been shown that when wines are exposed to air or oxygen they can develop the off-flavor. The mechanism for this process is unknown.

CHEMICAL NATURE OF MOUSY OFF-FLAVOR

Three chemical compounds have been identified as being responsible for mousy off-flavor in wine: ETHP, 2-acetyltetrahydropyridine (ATHP), and 2-acetylpyrroline (APY) (*10*−*12*, *32*, *37*, *38*).

Generally, a mousy wine sample will contain more than one of the causative compounds (*32*, *33*, *37*, *39*); however, no investigations have been undertaken to determine the sensory interactions between mousy off-flavor compounds. When pure, all three chemicals have an odor often described as "roasted" and "cracker-like" (*10*, *12*, *40*).

ETHP. The first report associating ETHP (**Figure 1**) with mousy off-flavor was in 1973 when it was detected using GC/MS in a mousy apple cider sample (*41*). In 1977, ETHP

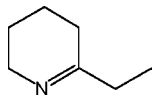

Figure 1. Structure of 2-ethyltetrahydropyridine.

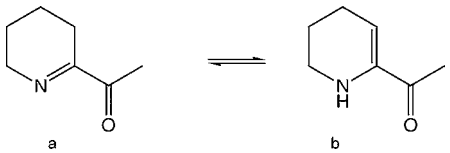

Figure 2. Tautomers of (**a**) 2-acetyl-3,4,5,6-tetrahydropyridine and (**b**) 2-acetyl-1,4,5,6-tetrahydropyridine.

was reported for the first time in a commercial wine (*38*); however, further studies were not able to confirm its presence in wine (*10*, *40*), resulting in its importance to mousy off-flavor in wine being questioned. More recent work has found that ETHP is indeed present in mousy wine samples (*32*, *39*).

ETHP occurs in tautomeric forms; however, the second tautomer (**Figure 1**) contributes only slightly to the total amount of the compound (*33*). The odor threshold of ETHP in wine is 150 μg/L (*40*); yet, until recently, it had only been detected in wine at levels substantially lower than this threshold (*32*). For this reason, there is a significant lack of studies on ETHP, as it was not considered a major contributor to mousy off-flavor in wine. It has now been established that ETHP can be produced at levels as high as 162 μg/L by certain strains of lactic acid bacteria (LAB) (*39*), making it an important mousy off-flavor contributing compound.

Grbin (*42*) speculated that the presence of ETHP in a *Dekkera*/*Brettanomyces*-infected mousy wine may be the result of a slow metabolic transformation of ATHP to ETHP. This was concluded after a study found that the formation of ETHP by *D. anomala* was delayed with respect to ATHP production and appeared to coincide with a decrease in the overall concentration of ATHP in a chemically defined medium. These results indicate that a critical concentration of ATHP appears necessary to stimulate the formation of ETHP, as a maximum concentration of ATHP was achieved before significant amounts of ETHP were detected (*42*). As ETHP takes longer to form than ATHP, with maximum production occurring well into the stationary phase (*42*), the importance of this compound with respect to its contribution to mousy off-flavor may have been underestimated due to the duration of experiments not being sufficient for metabolic transformation.

ATHP. ATHP (**Figure 2**) was first isolated in mousy wines in 1984 (*10*), where it was found to only be present in mousy and not sound wines. Its contribution to the mousy off-taste in wine has since been confirmed (*11*, *32*, *39*). ATHP is an oxidatively unstable compound (*43*, *44*), which is one of the reasons why, until recently, comparatively few wine-based chemical analyses have been undertaken on this compound. The detection threshold for ATHP in water is about 100 times lower than that of ETHP, at 1.6 μg/L (*45*), and it has been isolated in wine at levels of 4.8−106 μg/L (*32*).

ATHP, having a similar core structure to ETHP, exists in two tautomeric forms (2-acetyl-3,4,5,6-tetrahydropyridine and 2-acetyl-1,4,5,6-tetrahydropyridine) (**Figure 2**). Due to the methods used to identify mousy off-flavor in wine, that is sensory evaluation (by various means, as described above), it is possible that the distribution of the two tautomeric forms is pH-dependent, favoring the amino form (**Figure 2b**) under acidic wine conditions; however, it is the imino form (**Figure 2a**) that has a mousy off-flavor. An examination of this

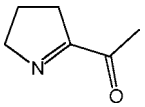

Figure 3. Structure of 2-acetylpyrroline.

tautomerism could explain why mousy off-flavor cannot be detected by simply sniffing the wine sample.

In wine, the low pH environment favors the more polar (and therefore less aqueous volatile) amino form of the tautomeric pair. Mouth saliva contains sodium bicarbonate, a mild base (*46*). It is possible that when wine comes into contact with saliva the tautomeric balance of ATHP shifts, favoring the more volatile imino form (**Figure 2a**), potentially explaining why mousy off-flavor cannot be smelled in a wine but becomes apparent when tasted. It is also this pH dependency of ATHP tautomerism that underpins the theory of both the palm and sniff method (as skin is only mildly acidic in comparison to wine, causing a pH shift) and the alkaline strip method, where sodium hydroxide on the strip causes the rapid alkalization and hence volatilization of the mousy compound.

ATHP is an important odorant and volatile flavor component in a number of foodstuffs including freshly baked bread (*43*, *44*, *47*), crackers (*43*, *48*), taco shells and corn tortilla chips (*49*, *50*), popcorn (*51*−*53*), and rice cakes (*54*). In these products, ATHP is described as having a "cracker biscuit" (*44*, *47*, *50*, *55*) or "roast-smelling" aroma (*52*, *53*, *56*, *57*). It is interesting to note that some individuals have associated this off-flavor as reminiscent of cracker biscuit (*34*).

Matrix effects or how components interact with each other within a particular matrix can explain the varying descriptors of ATHP in different substances. The majority of studies on ATHP have been in low water activity foodstuffs; however, wine has a high water activity and thus constitutes a very different matrix. Another factor that must be considered is the relative concentration of ATHP in these substances and the variability in taste perception between individuals.

APY. APY (**Figure 3**) was first identified in wine using GC/MS and GC-SNIFF techniques (*12*), when it was reported as being a major contributor to mousy off-flavor. The aroma impact of APY is an order of magnitude greater than that of ATHP, with the former's detection threshold in water being 0.1 μg/L (*58*). APY is a relatively unstable compound (*42*), which has been detected in wine in trace quantities up to 7.8 μg/L (*32*).

APY is responsible for the mousy aroma in wetted ground pearl millet (*Pennisetum americanum*) (*59*), and this is the only example in the literature that associates APY with mousy off-flavor, other than in wine. The two main food substances in which APY has been found to be a key odorant and flavor component are bread (*60*−*65*) and rice (*48*, *58*, *66*−*72*), particularly the more aromatic varieties such as Indian Basmati (*58*), where it is found at levels 10-fold above those of the common varieties (*73*). APY has also been identified in pandan leaves (*Pandanus amaryllifolius* Roxb.) (*73*), which are cooked in India and other parts of Asia with common rice varieties to impart an aroma that resembles the aroma of the more costly "scented" rice varieties. The concentration of APY in pandan leaves is 10-fold higher than in scented rice varieties and 100-fold that of common rice varieties. APY is an aroma compound in a variety of other products including rice cakes (*54*), boiled trout (*74*), canned, frozen, and fresh sweet corn (*75*), cooked lean beef (*76*), dried cured hams (*77*, *78*), lobster tail meat (*79*), and green tea (*80*) and has tentatively been identified in tiger urine (*81*).

APY's aroma has been described as "roasted" (*53*, *56*, *57*,

65, *76*, *82*) and "popcorn-like" (*49*, *73*, *74*, *83*). The variation in descriptors for APY (mousy, popcorn-like, and roasty) could be due to the relative concentration of the compound, matrix effects, and individual's variation of perception (*12*).

MICROBIOLOGY OF MOUSY OFF-FLAVOR

The microorganisms, *Dekkera*/*Brettanomyces* yeast and LAB, are generally associated with postprimary fermentation spoilage of wine. As well as being capable of producing mousy off-flavor, both organisms can have other effects, both positive and negative, on wine. *Dekkera*/*Brettanomyces* is generally regarded as a spoilage yeast, whereas certain strains of LAB are responsible for malolactic fermentation (MLF), as well as spoilage.

***Dekkera*/*Brettanomyces*.** *Brettanomyces*, and its sporulating equivalent *Dekkera*, have long been recognized as a fermented beverage spoilage yeast (*11*). Peynaud and Domercq (*6*) were some of the first to isolate these yeasts from mousy wine. Heresztyn (*11*) identified three strains of *Brettanomyces* as responsible for mousy off-flavor in wine. A more recent study confirmed that all species of *Dekkera*/*Brettanomyces*, including *D. bruxellensis*, *D. anomala*, *B. custersianus*, *B. nanus*, and *B. naardenensis*, can produce mousy off-flavor. This study also found that these species are capable of producing ATHP and ETHP, with the relative concentrations of these mousy heterocycles being strain-dependent (*32*).

Dekkera/*Brettanomyes* can have many and varied effects on wine. Sensory descriptors include cider, clovelike, spicy, plastic, smoky, medicinal, horsy, wet wool, band-aid, and mousy (*2*, *84*, *85*). In wine, this yeast has also been linked to an increase in turbidity (*8*, *84*), volatile acidity in general, and acetic acid production specifically (*11*, *84*). Why *Dekkera*/*Brettanomyces* has variable effects on wine is unclear. Presumably, the complex nature of wine plays an important role in this respect. However, the different characters produced may be dependent upon the concentration of specific precursors present in the wine and grape varietal components.

There is disagreement over the origin of *Dekkera*/*Brettanomyces* in wine. Yeasts depend on aerosols, human activity, or animal or insect vectors for their natural dispersal. The most common theories regarding yeast mobility incorporate soil, air, grapes, insect vectors, and cooperage. There is conflicting evidence in the literature as to whether *Dekkera*/*Brettanomyces* can be found on the grapes themselves (*2*, *86*, *87*). There are two main reasons why it is unlikely that the yeast will be isolated from the fruit in the vineyard. First, *Dekkera*/*Brettanomyces* require reasonably complex sources of exogenous nutrient including vitamin supplementation of which proliferation would be limited in clean fruit. Second, it is difficult to isolate a minority population from mixed flora that contains more superior and/or faster growing species (*2*).

There has been speculation that insect vectors could be responsible for the spread of *Dekkera*/*Brettanomyces*. The yeast has been found to be present in the breeding and feeding areas within the winery of the common winery insect, vinegar fly (*Drosophilia*) (*2*, *84*, *88*−*90*). The yeast, along with other microorganisms, is a normal part of its diet and it is able to adhere to the body, legs, and wings of the vinegar fly during foraging (*2*, *90*). Under laboratory conditions, *Dekkera*/*Brettanomyces* yeast was recovered externally from vinegar flies 24 h after feeding on the yeast (*90*), suggesting that vinegar flies may contribute to the dispersal of the yeast around a winery as a mechanical vector. *Dekkera*/*Brettanomyces* has also been isolated from the honey stomach of pollinating bees (*9*).

Oak cooperage can provide an ecological niche for the yeast as the cellobiose present in the charred oak wood can be used as a carbon source by the yeast (*2*, *20*). New cooperage contains more cellobiose than used cooperage and, therefore, can have a stimulatory effect on the growth of the yeast if contamination occurs. Once *Dekkera*/*Brettanomyces* has been introduced into the winery, it is speculated that it will build up in sites that are hard to clean, such as winery equipment (*2*, *90*). For this reason, the transfer of both must and juice can also spread *Dekkera*/*Brettanomyces* throughout wineries.

Molecular sulfur dioxide is toxic to *Dekkera*/*Brettanomyces* and therefore can be an effective inhibiting agent for the growth of this microorganism if maintained at the required levels. Although it is toxic to the yeast at low levels, sanitizing barrels with a sulfur dioxide solution or other antimicrobials is not totally effective against yeast infection due to the natural porosity of wood, in combination with the yeast's ability to live in the cracks of wine barrels and around the bung hole (*2*, *6*, *84*, *90*, *91*). For this reason, mousy off-flavor has a higher occurrence in wines that come into contact with barrels and are low in sulfur dioxide (*36*). The trend within the wine industry to move toward minimal use of sulfur dioxide in wine could be related to the increase in the number of cases of mousy off-flavor reported (*32*).

Dekkera/*Brettanomyces* can metabolize fermentable sugars; hence, a high concentration of glucose in wine enhances its growth rate. However, the yeast can develop substantial populations in a wine that is considered "dry", that is, low in residual sugar (*8*, *84*, *92*, *93*). For this reason, *Dekkera*/*Brettanomyces* has the ability to grow in bottled wine.

One autolysis product of *Dekkera*/*Brettanomyces* is glucose. This released glucose can then become a carbon source for the remaining viable population. For this reason, it may be risky for a winemaker to keep red wine on lees, even though this practice is speculated to improve wine quality (*93*).

LAB. LAB are a part of the natural microflora of wine. These bacteria have been found to enter the winery on the grapes and vine leaves. Wine can also be inoculated with LAB from commercial cultures to aid MLF. LAB are also spoilage microorganisms in wine. They have successfully adapted so as to be able to tolerate the physiochemical conditions of wine, such as low pH, the presence of ethanol and sulfur dioxide, and low temperatures. As well as being one of the organisms responsible for mousy off-flavor, they have also been known to cause mannitol taint, ropiness, acidification, acetification, bacterial haze and/or deposit, bitterness, and acrolein production (*7*, *39*, *94*, *95*).

LAB, in particular *Lactobacillus hilgardii*, were first linked to mousy off-flavor in Californian wines (*96*). *L. hilgardii* has since been confirmed as being one of the species responsible for causing mousy off-flavor in wines (*11*, *28*, *36*, *39*). This organism has been shown to be capable of producing ATHP at levels as high as 508 μg/L in an ethanolic grape juice medium (*39*), which is substantially higher than the odor detection limit of the compound. Other species have also been reported to be capable of causing mousy off-flavor in wine, including *L. brevis*, *L. buchneri*, *L. cellobiosis*, a *Pediococcus* species, and *Leuconostoc mesenteroides* (*11*, *36*, *39*).

As *Oenococcus oeni* is the preferred organism for the induction of MLF, it is of particular importance to winemakers that strains of *O. oeni* have been found to be capable of producing strong mousy off-flavor in a grape juice medium (*36*, *39*). Five strains have been shown to produce all three mousy heterocycles, including ETHP at concentrations higher than that

of other LAB strains, with one strain producing sensorially detectable concentrations (*39*). This supports the observation of Vaughn (*7*) who determined that *Leuconostoc* spp. (now reclassified as *Oenococcus*) could cause mousy off-flavor by metabolism of glucose and fructose under anaerobic conditions. Further studies are necessary to ascertain the abilities of *O. oeni* strains to produce mousy off-flavor compounds during MLF, under wine conditions. From this information, it has been concluded that mousy off-flavor formation is restricted to heterofermentative bacteria, and the general order of magnitude of N-heterocycle formation by LAB is *Lactobacillus* (heterofermentative) > *Oenococcus* > *Pediococcus* and *Lactobacillus* (homofermentative) (*39*).

As heterofermentative, as opposed to homofermentative, strains of LAB are capable of producing mousy off-flavor, this indicates that the respective pathway of sugar metabolism may play a key role in the occurrence of this spoilage (*39*). D-Fructose, a fermentable carbohydrate, has been directly linked to mousy off-flavor production, with the production being proportional to the concentration of D-fructose consumed by *L. hilgardii*. When D-fructose is omitted, the concentrations of APY and ATHP were comparatively low; however, ETHP production was not affected by D-fructose concentration (*33*).

AAB. AAB have been isolated from grapes, oak barrels, and inside wineries (*95*). AAB are vital to commercial vinegar production; however, they are also associated with wine spoilage, such as volatile acidity and occasionally mousy off-flavor production, although the research has been limited (*7*, *18*, *19*). Spoiled grapes harboring AAB and fungi have been found to stimulate the growth of LAB in wine (*95*).

FACTORS AFFECTING MOUSY OFF-FLAVOR PRODUCTION

Although the biosynthetic pathway by which *Dekkera*/*Brettanomyces* and LAB produce mousy off-flavor compounds in wine is unknown, the conditions necessary for its production have been established. L-Lysine and L-ornithine are responsible for the ring formations of the three mousy heterocycles, and ethanol and acetaldehyde have been shown to be responsible for the acetyl side chain. The presence or absence of certain metal ions and oxygen has a substantial effect on off-flavor production.

Amino Acids. The presence of particular amino acids in wine is essential to the production of mousy off-flavor compounds, particularly ATHP and APY. Tucknott (*28*) found L-lysine to be essential for mousy off-flavor production by bacteria in an apple juice medium. Since this first report, several investigations have been undertaken to determine the role of amino acids in mousy off-flavor development by both LAB and *Dekkera*/*Brettanomyces* in wine.

L-Lysine and L-ornithine, both of which occur naturally in grape juice, are necessary for the formation of mousy off-flavor by LAB. The addition of L-lysine increases the production of ATHP; however, the addition of L-ornithine increases the production of APY but represses the production of ETHP and ATHP. When both amino acids are added together, the relative concentrations of both APY and ATHP increase dramatically. Costello and Henschke (*33*) concluded that L-ornithine and L-lysine are responsible, at least for bacterial-produced mousy off-flavor, for the ring formations in APY and ATHP, respectively (*33*).

The amino acid L-lysine is also essential for the production of mousy off-flavor in wines affected with *Dekkera*/*Brettanomyces* (*11*, *28*, *32*, *97*). L-Lysine has a stimulatory effect on the production of mousy off-flavor compounds, especially ATHP. In the presence of L-lysine, all strains examined could produce ETHP but to a much lesser extent than that of ATHP; no APY was detected. Certain strains were capable of producing sensorially detectable mousy off-flavor in the absence of L-lysine (*32*), possibly due to the presence of endogenous L-lysine, as *Dekkera*/*Brettanomyces* have been shown to be capable of synthesizing L-lysine (*98*).

The uptake of L-lysine in a chemically defined medium corresponds with the initiation of ATHP production. As the strain of *D. anomala* progressed through the growth cycle from early exponential to stationary phase ATHP concentration increased, suggesting a link between mousy off-flavor production and associated growth metabolism. Cell numbers were not affected by the presence or absence of L-lysine so this did not account for the differences in concentration of ATHP (*97*).

As little as 10 mg/L of L-lysine can produce substantial concentrations of ATHP by *Dekkera*/*Brettanomyces*. No dose−response relationship has been established between L-lysine and ETHP concentration, despite ETHP only being detected when L-lysine is present (*97*). A nonproportional relationship between L-lysine concentration and ATHP production exists, in that this biotransformation is not efficient. This suggests that the biosynthesis of these off-flavor compounds is not the primary pathway of L-lysine catabolism, at least under the conditions of the experiment performed (*97*).

A feeding experiment utilizing *Dekkera*/*Brettanomyces* and uniformly labeled L-lysine $^{13}C_6$-$^{15}N_2$ showed that L-lysine is responsible for the synthesis of the tetrahydropyridine ring of ETHP and ATHP. In these molecules, five ^{13}C and one ^{15}N were incorporated into ATHP and ETHP molecules. Using single ^{15}N-labeled L-lysine revealed that the ϵ-nitrogen of L-lysine was incorporated into the ATHP ring, indicating that the α-amino group was removed (*97*). The mechanism by which this occurs was not investigated. The removal of the α-amino group is unique in the reported catabolism of L-lysine in nature.

The labeling experiments also show that the acetyl side chain of both ATHP and ETHP does not originate from the amino acid L-lysine, as there were no labeled carbons present in the side chain. This is supported by previous work (*11*, *28*, *33*). A simple retrosynthetic analysis of the ATHP molecule likewise does not support the lysine moiety as the side chain precursor.

When L-lysine was replaced with L-ornithine in a chemically defined medium, *D. anomala* was capable of producing APY. The concentration of L-ornithine (1000 mg/L) required to produce sensorily significant amounts of APY is higher than that which is found in grape juice or wine. This indicates that *Dekkera*/*Brettanomyces* yeasts are not responsible for the production of APY in wine. Therefore, detection of APY in a mousy beverage may be an indicator of bacterial spoilage (*97*). Further evidence for this is provided by an earlier study that showed that LAB could produce sensorily significant levels of APY in wine in the presence of L-ornithine (*33*).

L-Lysine has been shown to be responsible for the C_5N backbone of *Dekkera*/*Brettanomyces*-generated ATHP, suggesting that the biosynthesis of this compound may occur via a Δ^1-piperideine intermediate. It is possible that LAB-generated ATHP probably occurs by the same mechanism, as has been previously suggested (*33*). In a similar manner, L-ornithine may be found to be responsible for the C_4N backbone of LAB-generated APY, which may occur via a Δ^1-pyrroline intermediate. Further studies are required to further elucidate this relationship.

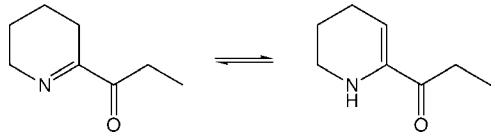

Figure 4. Tautomeric forms of 2-propionyltetrahydropyridine.

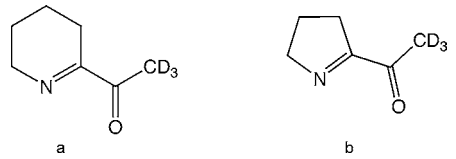

Figure 5. Deuterated forms of (**a**) 2-acetyltetrahydropyrdine and (**b**) 2-acetylpyrroline.

Ethanol. Ethanol is a necessary precursor for mousy off-flavor to occur in wine (*11*, *28*, *32*). In the absence of ethanol, mousy off-flavor is not produced (*11*, *38*, *99*, *100*) and this explains why mousy off-flavor occurs in wines infected with LAB post-alcoholic fermentation.

The additions of various alcohols to a medium containing *Lactobacillus* have been investigated to assess their effect on the formation of mousy off-flavor. With the addition of ethanol, strong mousy off-flavor is produced, *n*-propanol produces a low level of off-flavor, but 2-propanol, *n*- or *iso*-butanol, 2- or 3-butanol, and *n*-hexanol did not produce any (*28*), suggesting the alcohol present has to be a short chain primary alcohol. In the presence of *n*-propanol, both *D. bruxellensis* and *L. hilgardii* produced 2-propionyltetrahydropyridine (ATHP's propionyl analogue) (**Figure 4**) and ATHP (*11*, *28*, 33). ATHP is expected to occur as the yeast, and heterofermentative bacteria have the ability to produce ethanol through glucose metabolism (*11*). This suggests that ethanol is responsible for the acetyl side chain of ATHP and indicates an acetylation step is possibly involved in the biosynthesis. To further support the hypothesis that ethanol is responsible for the acetyl side chain of ATHP, it was found that *L. hilgardii* produced a deuterated form of ATHP in the presence of d_6-ethanol (**Figure 5a**), incorporating three deuterium atoms into the acetyl side chain.

When ethanol at 5% (v/v) was replaced with *n*-propanol in a medium containing *L. hilgardii*, no propyl homologue for APY (2-propionyl-1-pyrroline) or ETHP (2-propyltetrahydropyridine) was produced and neither was APY or ETHP (*33*), indicating that ethanol is not responsible for the side chain in these molecules or that the same mechanism is not valid for higher alcohols. However, a deuterated isotope of APY (**Figure 5b**) was produced with d_6-ethanol incorporating three deuterium atoms into the side chain of the molecule, indicating ethanol as the precursor to the acetyl side chain. In light of this, why no propyl analogue of APY was produced when *n*-propanol was present is unknown.

Although ETHP was found not to form when ethanol was substituted with *n*-propanol by *Dekkera*/*Brettanomyces* (*11*) or form a deuterated equivalent in the presence of d_6-ethanol, trace quantities of ETHP and a compound tentatively identified as 2-propyltetrahydropyridine were found to form, by LAB, in the presence of 2-propanol (*33*). Neither ATHP or APY was formed in the presence of this alcohol (*33*).

Ethanol appears to be the precursor to the side chain of both APY and ATHP by LAB and *Dekkera*/*Brettanomyces*. Although experiments indicate that it is not directly responsible for the side chain of ETHP, it further suggests that ETHP is formed as a result of ATHP metabolism. Interestingly, 2-propanol stimulates the production of ETHP.

Aldehyde. Acetaldehyde is also a precursor to the side chain of LAB-produced ATHP. The production of APY and ATHP by *L. hilgardii* is stimulated with the addition of acetaldehyde in a chemically defined medium. However, the elimination of acetaldehyde has only a slight effect on APY and ATHP production. When propionaldehyde or butyraldehyde were added, there were no detectable amounts of C-3 or C-4 substituted homologues of ETHP, ATHP, or APY produced. When d_4-acetaldehyde was introduced, minor quantities of ATHP containing three deuterium atoms in the acetyl side chain were produced (**Figure 5a**). This suggests that acetaldehyde plays a part in the formation of the side chain of the ATHP molecule. The deuterated form of acetaldehyde had no effect on the formation of ETHP or APY (*33*). No studies have been conducted examining the effect of acetaldehyde on *Dekkera*/*Brettanomyces*-produced mousy off-flavor.

Metal Ions. The presence and/or absence of certain metal ions in wine has an effect on the formation of mousy compounds by LAB. The elimination of Fe^{2+} (as $FeSO_4 \cdot 7H_2O$), Mg^{2+} (as $MgSO_4 \cdot 7H_2O$), Mn^{2+} (as $MnSO_4 \cdot H_2O$), and Ca^{2+} (as $CaCl_2 \cdot 2H_2O$), originally present at levels of 25, 25, 43, and 1660 mg/L respectively, from a chemically defined medium prevented the formation of APY and reduced the formation of ATHP by 96% and ETHP by more than 50%. In particular, the elimination of Fe^{2+} substantially decreased the concentration of APY and ATHP (94%), whereas the elimination of Mn^{2+}, Mg^{2+}, or Ca^{2+} caused only a small reduction in their concentrations. The elimination of Fe^{2+} also decreased the production of ETHP (90%). The exclusion of Mn^{2+} or Mg^{2+} did not have a significant effect on ETHP production; however, the omission of Ca^{2+} doubled the production of the compound (*33*).

This information suggests that a sufficient amount of ferrous ions is one of the major physicochemical factors necessary for mousy off-flavor development in wines. However, its actual role in biosynthesis is unclear. The idea that a sufficient amount of "active" iron is necessary for mousy off-flavor to occur in wines is not new: It has been speculated since the 1950s (*27*, *101*). There have been no investigations of the effect of metal ions on the production of mousy off-flavor by *Dekkera*/*Brettanomyces*, and further studies are necessary to fully elucidate the role of trace elements in the biosynthesis of mousy compounds.

Oxygen. Oxygen can cause wine to become mousy; however, the mechanism by which this occurs is unknown. It has been hypothesized that oxygen may have a direct effect on the oxidation state of the mousy off-flavor molecules themselves (*32*). The majority of the work on *Dekkera*/*Brettanomyces* and LAB associated mousy off-flavor has been in the presence of air. Tucknott (*28*) was the first to suggest that oxidation is an important factor in the production of mousy off-flavor by LAB and *Dekkera*/*Brettanomyces*; however, the relationship was not investigated.

Aeration has been shown to stimulate the growth of *Dekkera*/*Brettanomyces* in wine (*93*). Additionally, a unique characteristic of *Dekkera*/*Brettanomyces* is that alcoholic fermentation is stimulated by molecular oxygen but inhibited under strict anaerobic conditions. This is termed the "Custers effect" (*102*).

Oxygen has a stimulatory effect upon the production of both ATHP and ETHP by *D. bruxellensis* (*42*). The relative concentrations of these two molecules were produced at levels significantly higher in air-saturated conditions than under air-limiting conditions when grown in grape juice medium. However, the biomass produced was greater in the air-saturated treatment also (*42*); therefore, this could account for the difference. The production of ATHP by *D. bruxellensis* under

air-saturated conditions was higher than that of ETHP. However, under air-limited conditions, more ETHP was detected than ATHP. Although the reason for this trend could be due to the presence of oxygen, it could also be due to the duration of the experiment being sufficiently long enough to allow for a slow metabolic transformation of ATHP into ETHP (*42*).

In a *D. anomala* high-density whole cell assay system, the anaerobic incubation of anaerobically precultured cells was shown to repress ATHP formation. However, if the anaerobic precultured cells were transferred to an aerobic incubation condition, ATHP production was stimulated. This suggests that oxygen may be influencing off-flavor production directly, not just by stimulating growth and biomass formation. Thus, oxygen may be directly involved in the biosynthesis of off-flavor compounds via an oxygen-dependent enzyme system or an oxidative chemical mechanism. When coupled with the requirement of LAB for the presence of ferrous ions for mousy-off flavor formation, these two factors must be viewed as extremely significant. However, off-flavor production was not strongly repressed under aerobic preculturing followed by anaerobic incubation. The reason for this is unknown. It was suggested that aerobic preculturing could influence the cell physiology by predisposing or adapting the yeast for taint production, which then occurred in the presence of the appropriate precursors irrespective of the subsequent anaerobic incubation condition. It was suggested that oxygen might be the "switch" for mousy off-flavor biosynthesis by yeast (*42*).

Oxidative metabolism may play a role in providing a metabolic process that facilitates the biosynthesis of compounds. Further work is needed on the influence of oxygen to elucidate the relationship. No work has been undertaken into the effect of oxygen and LAB-generated mousy off-flavor.

From the information gathered pertaining to the factors necessary for LAB to produce the mousy heterocycles ATHP and APY, Costello and Henschke (*33*) were able to propose a pathway of formation (**Figure 6**). The authors have stated that further work must be undertaken to confirm this pathway. There have been insufficient data collected to propose a pathway of formation for LAB-produced ETHP. Thus, a pathway has been proposed for *Dekkera*/*Brettanomyces*-produced ATHP and ETHP (**Figure 7**).

OTHER FORMS OF MOUSY OFF-FLAVOR?

Anecdotal evidence suggests that there may be other forms of mousy off-flavor in wine. When examining LAB-produced mousy off-flavor, it was discovered that the level of off-flavor fluctuated during the growth of the microorganisms, with maximum taint detected during the early stages of incubation and the intensity diminishing toward the end (*36*). This indicates that there may also be a transient, strain-dependent form of mousy off-flavor that can occur during the course of MLF.

It has been noted anecdotally (*103*) that there appears to be a transient form of mousy off-flavor that can occur in wine with different sensory characteristics to currently known mousy heterocycles. This form, however, does not appear to be related to either LAB or *Dekkera*/*Brettanomyces*.

In conclusion, it can be speculated that the mechanism by which LAB and *Dekkera*/*Brettanomyces* form mousy off-flavor in wine is similar. However, more research needs to be undertaken before this can be ascertained.

To prevent the biosynthesis of the mousy off-flavor-forming compounds, elimination or strict control of the causative yeast and bacteria must be maintained. This can be achieved by implementing microbial control strategies in the winery. When

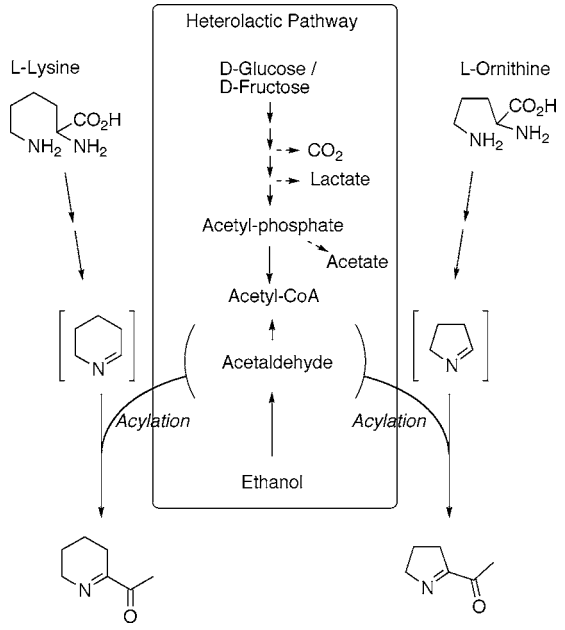

Figure 6. Proposed pathway by Costello and Henschke (*33*) for the formation of the mousy heterocyles, APY (2) and ATHP (3), by the heterofermentative lactic acid bacterium, *L. hilgardii* DSM 20176. Compounds in bold type (D-glucose, D-fructose, ethanol, acetaldehyde, L-lysine, and L-ornithine) are considered key substrates. The reaction was also dependent upon the presence of ferrous ions. Reprinted with permission from ref *33*. Copyright 2002 American Chemical Society.

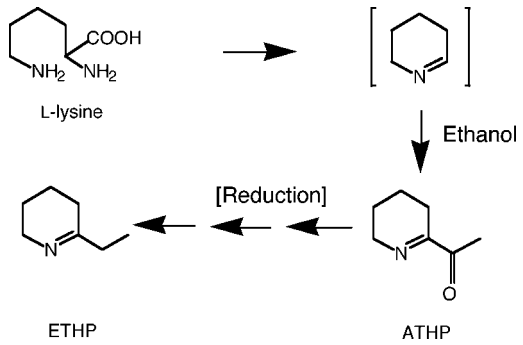

Figure 7. Proposed pathway for the formation of the mousy heterocycles ATHP and ETHP by *Dekkera*/*Brettanomyces* in wine.

the growth of the bacteria is being encouraged to aid MLF, careful strain selection, preliminary trialing, and constant monitoring should be undertaken. Research is currently being undertaken examining possible methods of removal of the mousy off-flavor molecules once they have formed in wine (*104*).

ABBREVIATIONS USED

AAB, acetic acid bacteria; APY, 2-acetylpyrroline; ATHP, 2-acetyltetrahydropyridine; ETHP, 2-ethyltetrahydropyridine; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; LAB, lactic acid bacteria; MLF, malolactic fermentation; rH, redox potential.

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NOTE ADDED AFTER ASAP PUBLICATION

A sentence has been added to the Introduction after the original posting of August 8, 2006. Several typographical errors have also been corrected in the posting of August 16, 2006.

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