IVES Technical Reviews
vine & wine

https://doi.org/10.20870/IVES-TR.2023.7867

Simultaneous assay of mousy off-flavor markers in wine

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The compounds described as being responsible for mousy off-flavor in wine are 2-acetylpyrroline (APY), 2-acetyltetrahydropyridine (ATHP) and 2-ethyltetrahydropyridine (ETHP). However, assay of these compounds is difficult due to their physical-chemical characteristics. The aim of this study was to develop a simple and effective method for simultaneously determining the concentration of these three *N*-heterocyclic compounds in wine. This new method is a decisive tool for better defining this spoilage phenomenon in wine and guarding against it.

Introduction

Mousy off-flavor is a wine fault of microbial origin. It is described as particularly unpleasant, reminiscent of urine or a mouse cage, but also and most often grilled foods such as popcorn, crackers and bread crust, rice, or sausage skin[1 2 3]. While this type of spoilage may have been misnamed in olfactory terms, it appears more clearly on the palate. The *N*-heterocyclic compounds involved are not very volatile at wine pH[4]. The pH in the mouth is higher than in wine, which explains why this fault is perceived more strongly on the palate via retro-olfaction. Described by Peynaud and Domercq[1] as early as 1956, this fault was thought to be linked to the presence of *Brettanomyces*-type yeasts, according to Australian professionals[2 4]. However, before the 2010s, mousy off-flavor was an extremely rare fault. It has become more frequent in recent years, associated with an increase in wine pH, as well as with winemaking practices that tend to significantly reduce the use of sulfur dioxide (SO_2). Until now, quantitative data for mousy off-flavor markers has been limited due to the analytical challenges associated with the nature of these compounds and their low concentrations, in the order of µg.L^{-1}. Concentrations previously reported in wine range from 0.7 to 106 µg.L^{-1} for ATHP[5]; for APY, a concentration of 7.8 µg.L^{-1} has been reported by Snowdon *et al.*[2], while ETHP concentrations found in wine can exceed 150 µg.L^{-1} [4]. There are currently no detection thresholds for these compounds (though they would be very useful), as measurements have been carried out using different sensory protocols and in different matrices[3], making comparison difficult. With the aim of filling this gap and understanding the parameters influencing the appearance of mousy off-flavor, this study has developed a simple and effective method for simultaneous assay in wine of the three *N*-heterocyclic compounds involved. Hence, a technique has been developed using Stir Bar Sorptive Extraction (SBSE, Twister®) followed by gas chromatography-mass spectrometry (GC-MS) analysis. The SBSE technique is already used for multi-fault assay of wine[6].

Compounds responsible for mousy off-flavor

The main compounds described as being responsible for mousy off-flavor in wine are 2-acetylpyrroline (APY), 2-acetyltetrahydropyridine (ATHP) and 2-ethyltetrahydropyridine (ETHP). These compounds have the particularity that they coexist in two tautomeric forms (the imine and enamine forms). The reactivity of the tautomeric forms with the protons of the medium implies a pH-dependent change in their volatility. It is important to characterize the presence of these compounds in their various forms, and to quantify them in order to perceive the reality of spoilage in wines beyond simple olfactory perception, which seems to vary greatly depending on the wine or its redox state. Their proven absence can be an important point when the presence of a fault is wrongly suspected, or to avoid any confusion.

Sensory detection and development of a standard method

The various sources of variability (origin of the markers, concentrations, specificity of the wine matrix, personal detection capabilities, composition and pH of the taster's saliva, for example) partly explain the lack of consensus regarding the perception of this fault in wine. Various sensory methods for detecting mousy off-flavor in wine have been developed, using alkaline paper strips or by pH adjustment. Among the methods tested, adjusting the pH of red wines to around 5 by adding sodium bicarbonate increases consensus among tasters, as well as their detection and discrimination ability[3].

Principle of the analytical method developed

The SBSE-Twister® extraction method has been optimized[7]. It was necessary to increase the pH of the sample. The aim is to shift the equilibria to non-protonated forms, which are less polar and easier to extract. Indeed, SBSE is well suited to the extraction of apolar analytes. Furthermore, the addition of sodium chloride (NaCl) improves the recovery of analytes by reducing their solubility in the aqueous phase and increasing their affinity for the apolar phase of the Twister®.
Hence, to prepare the sample, the pH is adjusted to 10.5 with an aqueous sodium hydroxide solution (NaOH, 1 M).

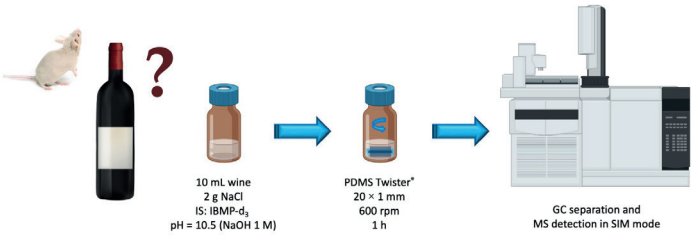


FIGURE 1. Analytical method using SBSE (IS: internal standard).

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TABLE 1. Performance of the analytical method (adapted from Kiyomichi *et al.* [7]).

Compound	Range (µg.L^{-1})	White wine			Rosé wine			Red wine		
		LOD [a] (µg.L^{-1})	LOQ [a] (µg.L^{-1})	Recovery (n = 3) (%)	LOD [a] (µg.L^{-1})	LOQ [a] (µg.L^{-1})	Recovery (n = 3) (%)	LOD [a] (µg.L^{-1})	LOQ [a] (µg.L^{-1})	Recovery (n = 3) (%)
APY	3.6 – 71.3	0.8	2.7	103	0.9	3.1	88	0.5	1.8	101
ATHP	5.3 – 105.0	0.8	2.6	104	0.9	3.2	100	1.5	4.9	93
ETHP	14.1 – 282.7	0.6	2	96	1.2	4.2	110	2.7	8.9	103

[a] LOD: limit of detection, LOQ: limit of quantification.

To 10 mL of the sample at pH 10.5, 2 g of NaCl is added. Once the NaCl has completely dissolved, the internal standard (IBMP-d_3) is added to the vial, along with a PDMS Twister® (Gerstel, 20 mm × 1 mm; length × phase thickness). After extraction with stirring at 600 rpm for 1 hour, the Twister® is removed from the sample and desorbed into the GC-MS for analysis (Figure 1).

In this method, the imine and enamine forms of ATHP are detected, unlike the enamine forms of APY and ETHP. Either the equilibrium is very strongly shifted towards the imine forms, or the extractability of the enamine forms by PDMS is low due to their chemical characteristics. The ATHP concentration is thus determined by summing the results for A-3,4,5,6-THP and A-1,4,5,6-THP. The limits of detection (LOD) and quantification (LOQ) of APY, ATHP and ETHP for white, rosé and red wines are given in Table 1.

Analysis of wine samples

The optimized method was applied to the analysis of commercial wine samples from different origins and produced using a variety of winemaking practices. This study included analysis of 6 wines produced with "conventional" use of SO_2 and 68 wines produced without addition of SO_2. Classification into the two categories, conventional or limited sulfite addition, was based on indications concerning the use of SO_2 on the label or from the producer. It has been demonstrated that almost all wines produced without SO_2 or with limited sulfite addition contain ETHP, whereas this compound is not quantifiable in wines where sulfites have been added. On the other hand, APY and ATHP were detected only in wines produced without SO_2 or with limited sulfite addition. In all, 11 wines contained APY and 39 wines contained ATHP (Figure 2). Concentrations were always below 7.7 µg.L^{-1} for APY, 54.5 µg.L^{-1} for ATHP and ranged from 14.0 µg.L^{-1} to 120.5 µg.L^{-1} for ETHP.

The results highlight several points. First, the conventional use of SO_2 could limit the appearance of the heterocyclic compounds responsible for mousy off-flavor. Secondly, the presence of APY is generally associated with the presence of the other two heterocyclic compounds.

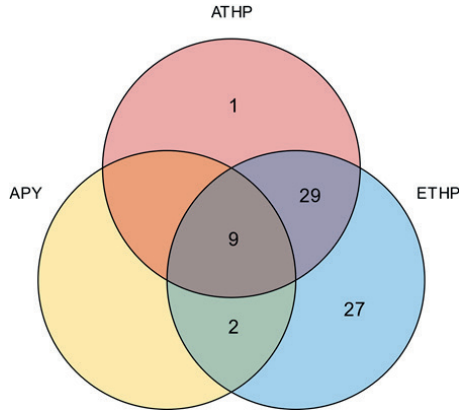


FIGURE 2. Distribution of the mousy off-flavor in the 74 wines analyzed (adapted from Kiyomichi *et al.*[7]).

Lastly, wines suspected of taint with mousy off-flavor may contain APY or ATHP, or both of these compounds, but sometimes neither (presence below the limit of detection). As for ETHP, it is present in almost all wines produced without SO_2 or with limited sulfite addition. Further analyses will be needed to confirm these initial results with more data and a more diverse range of samples.

Conclusion

A relatively simple method, accessible to laboratories with the necessary equipment, has been developed to determine the concentrations of the *N*-heterocyclic compounds responsible for mousy off-flavor. It consists in the use of SBSE or Twister® extraction followed by gas chromatography with mass spectrometry detection. This method makes it possible to check tasting results, to confirm the presence of the olfactory fault or, on the contrary, eliminate doubts when perception of the fault is difficult on tasting. In addition, the mechanism for generation of mousy off-flavor has yet to be fully elucidated. Several hypotheses point to very high microbial diversity[8] or a modification of the wine's redox balance. This new method is expected to contribute to a better understanding of this all-too-frequent fault. ■

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