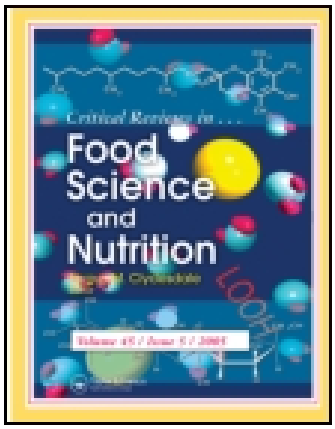

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Methoxypyrazine Analysis and Influence of Viticultural and Enological Procedures on their Levels in Grapes, Musts, and Wines

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Methoxypyrazine Analysis and Influence of Viticultural and Enological Procedures on their Levels in Grapes, Musts, and Wines

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This review discusses the factors that affect the concentrations of methoxypyrazines (MPs) and the techniques used to analyze MPs in grapes, musts, and wines. MPs are commonly studied pyrazines in food science due to their contribution of aroma and flavor to numerous vegetables such as peas and asparagus. They are described as highly odorous compounds with a very low olfactory threshold. The grape varietals that exhibit green or herbaceous aromas that are characteristic of MPs are predominantly Vitis vinifera *cv. Cabernet Sauvignon and Sauvignon Blanc, but include others. The most extensively studied MPs include 3-isobutyl-2-methoxypyrazine, 3-isopropyl-2-methoxypyrazine, and 3-sec-butyl-2-methoxypyrazine. It outlines the significance of methoxypyrazines in grapes, musts, and wines in terms of the concentrations that are capable of contributing their sensory characteristics to wines. This review discusses methods for analyzing MPs including gas chromatography-mass spectroscopy (one or two dimension) and high-performance liquid chromatography, the appropriate extraction techniques, and the efficacy of these methods. Additionally, this review explores factors that affect pyrazine content of grapes, must, and wines, such as the effects of different viticultural practices, effects of light exposure and grape maturation, climate, soil, the multi-colored Asian lady beetle and the effects of different vinification processes.*

Keywords Wine, grape, methoxypyrazines, aroma, flavor, *Vitis vinifera*, Cabernet Sauvignon, Sauvignon Blanc, nitrogen compounds

INTRODUCTION

Pyrazines are nitrogen-containing heterocyclic compounds found in many plant, and are most represented in food aromas (Vernin and Vernin, 1982). These heterocyclic compounds are extensively studied in the food industry because they impart an effect upon aroma. Methoxypyrazines (MPs) are commonly studied pyrazines in food science due to their contribution to the vegetative or earthy aromas of various fresh vegetables such as peas, asparagus, lettuce, potatoes, and others (Murray and Whitfield, 1975; Gallois, 1984; Allen et al., 1994). An early study by Buttery et al. (1969) associated MPs with bell pepper aromas, and additionally noted the extremely low sensory threshold of MPs. MPs are present in many plant species, but analysis has been performed in-depth mainly for wine grapes due to their ability to impact wine aroma and flavor. Certain *Vitis vinifera* cultivars exhibit characteristic "green" or "herbaceous" aromas that are associated with MPs. These varietals predominantly involve Cabernet Sauvignon and Sauvignon Blanc, but also include Cabernet Franc, Merlot noir (Augustyn and Rapp, 1982a; Augustyn et al., 1982b; Allen et al., 1988; Marais, 1994; Lopez et al., 1999; Hashizume et al., 2001; Sala et al., 2002,

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Table 1 Reported odor descriptions and olfactory thresholds (ng/L) of the three primary 3-alkyl-2-methoxypyrazines found in wine grapes and wine

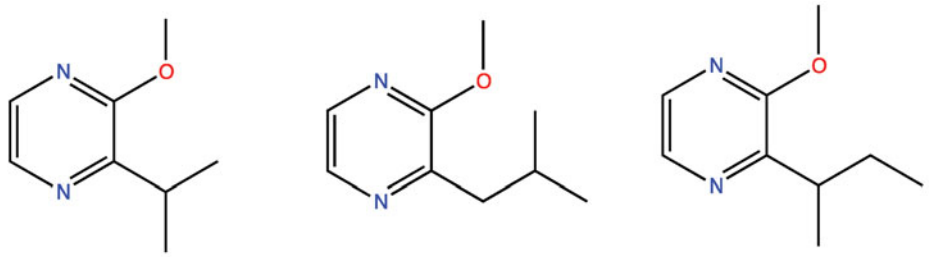
MP	Medium	Odor description	Threshold	Reference
3-isopropyl-2-	White wine	Earthy, cooked or canned asparagus, green beans	2	Allen et al., 1991
	Air	Earthy, roasted	0.0005–0.005	Boelens and Gemert, 1995; Blank et al., 1992
	Water	Galbanum, earthy, musty, potato, green pepper, roasted, peas, moldy, cellar	1 to 2	Maga, 1990; Boelens and Gemert, 1995; Kotseridis et al., 1999a; Seifert et al., 1970; Parliment and Epstein, 1973
	Olfactometry	Grassy, earthy		Hashizume and Samuta, 1997
	Red wine	Leafy	2	Maga, 1990
	Synthetic wine	Musty, earthy, leafy	2	Maga, 1990
3-sec-butyl-2-	Water	Green (peas, bell pepper, galbanum), ivy leaves, bell pepper	1 to 2	Boelens and Gemert, 1995; Mihara and Masuda, 1988; Parliment and Epstein, 1973; Murray et al., 1970; Murray and Whitfield, 1975
3-isobutyl-2-	Air	Earthy	0.002–0.005	Boelens and Gemert, 1995; Blank et al., 1992; Buttery et al., 1969
	Water	Green, bell pepper, musty, earthy	10	Boelens and Gemert, 1995
	Water	Green, bell pepper	2	Maga, 1990; Seifert et al., 1970; Parliment and Epstein, 1973; Buttery et al., 1969
	White wine	Fresh vegetables, bell pepper, green gooseberries, herbaceous, vegetative	1	Allen et al., 1991
	Olfactometry	Herbaceous, earthy		Hashizume and Samuta, 1997
	Water	Strongly green, bell pepper	16	Takken et al., 1975
	Water		0.5	Kotseridis et al., 1998a
	Red wine	Leafy	10, 15, 16	Maga, 1990; Roujou-de-Boubée et al., 2000; Kotseridis et al., 1998a
	Synthetic wine	Musty, green pepper	2, 6	Maga, 1990; Roujou-de-Boubée et al., 2000

2004), and many other varieties (Campo et al., 2005; Escudero, 2007; Koch et al., 2010).

Many media such as air, water, and synthetic wines have been used to determine odor descriptions and thresholds. MPs possess a very low olfactory threshold, which allows them to have a significant impact on food and wine aroma. Authors have reported many descriptions of these molecules ranging from a green, vegetative odor to a roasted or nutty note (Table 1, Buttery et al., 1969; Allen et al., 1994; Boelens and Gemert, 1995; Masuda and Mihara, 1988). The potency of these odorant compounds has led MPs to be some of the most odor-active compounds known (Dunlevy et al., 2009). The variation of descriptions for these compounds may be influenced by factors such as the evaluation process, medium influence, MP concentrations, environmental conditions, individual olfactory sensitivity differences, and procedural differences. MPs are also capable of providing varietal characteristics and aromatic complexity if they are complemented by other fruity and herbaceous aromas (Allen et al., 1988, 1995; Marais, 1994); however, these compounds easily reduce wine quality if it is the dominant aroma in the real wine headspace.

While most papers report a correlation between MPs concentrations and vegetal odors, a study published by Preston et al. (2008) could not find any. The authors used various wines with varying MPs levels, but most likely in combination with significant levels of other odorant compounds. The major factor distinguishing these wines is the contrast between the combined vegetal characteristics and the fruity characteristics. It is also possible that the MPs were "buried" or "hidden" due to interactions or interference with other odorant compounds.

In general, the classification of MPs is performed according to their origins: those formed by heat; those produced by microorganisms; and those naturally present in plants. Commonly identified MPs in the food industry as also in wines include 2-methoxypyrazine (2-MP), 3-methyl-2-methoxypyrazine (MEMP), 3-ethyl-2-methoxypyrazine (ETMP), 3-isopropyl-2-methoxypyrazine (IPMP), 3-isobutyl-2-methoxpyrazine (IBMP), and 3-sec-butyl-2-methoxypyrazine (SBMP) (Seifert et al., 1970, 1972; Takken et al., 1975; Sala et al., 2000). Especially in wines, the identified MPs are formed biochemically in the vines or as a contamination by lady beetles (Pickering et al., 2008a). IPMP, SBMP, and IBMP, in particular, are considered the most abundant in wines (Figure 1) (Bayonove et al., 1975; Murray and Whitfield, 1975; Maga, 1982, 1992; Gallois, 1984; Harris et al., 1987; Allen, 1993; Hashizume and Umeda, 1996). A number of researchers have published extensive reviews on MPs that display the aromatic characteristics of these odor active compounds (Shibamoto, 1980; Maga, 1982, 1992; Gallois, 1984; Rizzi, 1988; Sala et al., 2008a, 2008b). Given that these compounds have the ability to impact aroma and flavor at such a high intensity, understanding the conditions that influence their presence is an important concern for the wine industry. Since excessive vegetative or herbaceous notes are detrimental to high end wines, it is important to understand the factors that influence MP production and accumulation in wine grapes, and ultimately, the presence of these compounds in the produced wine (Arnold and Bledsoe, 1990; Colagrande and Fumi, 1993; Noble et al., 1995; Kotseridis et al., 1998a; Roujou-de-Boubée, 1999; Roujou-de-Boubée et al., 2000). The mechanisms of biosynthesis and accumulation of MPs and their



3-isopropyl-2-methoxypyrazine (IPMP)

3-isobutyl-2-methoxypyrazine (IBMP)

3-sec-butyl-2-methoxypyrazine (SBMP)

Figure 1 Chemical structures of the three most abundant MPs in grapes, musts and wines: IPMP, IBMP, and SBMP.

precursors have been suggested but there is still a considerable amount of work to be done (Dunlevy et al., 2010; Ryona et al., 2010).

IBMP is the most abundant MP and is most likely to affect wine aroma because it is often present in concentrations above its sensory threshold (Harris et al., 1987; Allen et al., 1991, Allen, 1993; Hashizume and Umeda, 1996). Nevertheless, levels of IBMP exceeding the sensory threshold may be well suited with the aroma milieu of high-end Cabernet Sauvignon and Merlot wines due to the complexity of these wines (Allen et al., 1995; Kotseridis et al., 1999b). The characteristic "bell pepper" aroma in Sauvignon Blanc and Cabernet Sauvignon wines has been correlated with IBMP levels (Allen et al., 1991; Noble et al., 1995; Rajchl et al., 2009). Wines that contain higher concentrations of IBMP have more of a vegetative aroma and are often linked to unripe grapes or poor quality wines (Roujou-de-Boubée, 1999; Roujou-de-Boubée et al., 2000). The sensory thresholds for IBMP differ from white, red and synthetic wines, as the value found were of 1–6 ng/L and 10–16 ng/L in red wines (Maga, 1990; Kotseridis et al., 1998a; Roujou-de-Boubée et al., 2000). The sensory threshold in water, 2 ng/L, is considerably lower than in wine due to the difference in complexity of the red and white wine matrices that tend to mask the aroma and results in higher thresholds (Maga, 1990). IPMP is the second-heaviest MP in wines; it has been described as a potato-, earthy-, or a pea/asparagus-like smell (Seifert et al., 1970; Allen et al., 1994). IPMP is found in grapes and wines, but has also been found to be produced by microorganisms and excreted by a common vineyard pest known as the "multi-colored Asian lady beetle" (*Harmonia axyridis*). The presence of this beetle on grapes results in higher amounts of IPMP in musts and wines as the beetle is present in the must in significant amount (Gallois et al., 1988; Pickering et al., 2005, 2007a, 2007b, 2008a; Cai et al., 2007; Kotseridis et al., 2008).

ETMP and SBMP have been identified in wines, but are typically not found in high enough concentrations to have an influence on wine aroma (Augustyn and Rapp, 1982a; Augustyn et al., 1982b; Allen et al., 1994). The sensory threshold for SBMP is 1–2 ng/L in water (Parliment and Epstein, 1973; Murray and Whitfield, 1975; Mihara and Masuda, 1988; Maga, 1992; Boelens and Gemert, 1995). Lacey et al. (1991) reported that ETMP was not detected in 22 wine samples obtained from Australia, New Zealand, and France. Allen et al. (1994) reported that SBMP may reach 2–6 ng/L in Cabernet Sauvignon and that the highest levels ever recorded were 4–16 ng/L in Merlot noir wines. In contrast, the sensory threshold for ETMP is 400–425 ng/L in water, which is significantly higher than the other MPs and as a result, it would not likely be present in a high enough concentration to impact wine aroma and flavor (Buttery et al., 1970; Seifert et al., 1970; Parliment and Epstein, 1973; Maga, 1990). Lacey et al. (1991) reported that ETMP was not detected in 22 wine samples obtained from Australia, New Zealand, and France. The data on these compounds is inadequate and further research must be conducted to understand their potency and contribution to wine aroma.

The first aim of this review is to discuss the analytical methods and instrumentation used for MPs over previous years, biosynthesis of MPs, their concentrations in grapes, musts and wines, their concentrations dependent upon aging and the storage period, and microorganism and insect influences on MP accumulation in wine. Techniques developed for control of MP concentrations in viticulture and winemaking are also discussed.

METHODS AND TECHNIQUES FOR ANALYSING MPs

Introduction to MP Analysis

MPs in grapes and wine typically occur at low concentrations (low ng/L levels), which makes analysis difficult. Compounding this problem is that the complexity of real wine headspace means that numerous other compounds may interfere, mask, or multiply the intensities of the aromas in the wine. Many methods to isolate, extract, purify, quantify, and identify MPs have been developed over the past few decades to cope with this issue, including rigorous steps to concentrate and purify the sample, which made the analyses time-consuming (Harris et al., 1987; Allen et al., 1994; Hashizume and Umeda, 1996; Kotseridis et al., 1999b). The sensitivity of analytical techniques has to be extreme to obtain accurate, yet reliable results. As researchers

became more involved in this field, new techniques were developed that yielded higher sensitivity and allowed MP analysis to become more accurate and less time consuming.

Current methods for overcoming the challenges involved with analysis of MPs in grapes, musts and wines involve modern extraction techniques paired with powerful detectors. Extraction methods such as liquid–liquid extraction (LLE), solid-phase extraction (SPE), headspace SPME (HS-SPME), and stir-bar sorptive extraction (SBSE) allow powerful instruments to detect MPs in concentrations on the ng/L scale. Quantitative analysis of these compounds have used combinations of gas chromatography-mass spectroscopy (GC-MS), liquid chromatography-mass spectroscopy (LC-MS), high-performance liquid chromatography (HPLC) for purification, gas chromatography combined with time-of-flight mass spectrometry (TOF-MS), or gas chromatography combined with nitrogen phosphorous detectors (NPDs), and other techniques.

Background

The most abundant MP, IBMP, was first reported in Cabernet Sauvignon musts by Bayonove et al. (1975). Since then, many researchers attempted to quantify this compound in wines; unfortunately, this is a difficult task as IBMP is generally present at low ng/L levels. As noted before, it is not only the extremely low concentrations of the targeted compounds, but also the matrix effects and the potent interferences with other compounds. The fact that MPs are present at very low detection thresholds, thus possibly influencing wine quality at very low concentration levels, requires the development of reliable quantitative methods for their concentration levels. Conversely, analytical methods should be robust to allow for the analysis of a large number of samples. Since the late 80s, several approaches have been essayed to quantify these compounds. The first methods used relatively large volumes of sample and the sample preparation procedures were complex but more recent procedures have been simplified by the use of SPE, SPME, and SBSE for sample preparation. It should be noticed that first sensitive, reproducible, and accurate method published on the analysis of MPs is that of Harris et al., 1987. The authors developed a highly sophisticated method using isotopically labeled MPs as internal standards, apparently one of the very first methods using deuterium labeled analogs to quantify odorant compounds in the wines.

Analysis in Wines vs. Grape Must Samples

The majority of reports have focused on measuring MPs in wines, but it is more useful to predict the final levels of MPs in the wine by analyzing the grapes, and allow the winemaker to decide upon the appropriate winemaking technology and techniques to control MPs in the final product (Ryona et al., 2009). Earlier attempts were not successful, probably due to heterogeneity of the grape samples and the time needed for MPs to be extracted from skins into the juice. Ryona et al. (2009) achieved a high correlation between MPs in grates and wine, and demonstrated that high temperatures or prolonged skin contact times are necessary to facilitate diffusion of IBMP from the skins into the juice; thus, incomplete or inconsistent extraction could explain the difficulties encountered in correlating IBMP levels in juice to IBMP levels in wine.

Table 2 Sample preparation, isolation of volatile fraction, and clean-up methods used for analyzing MPs

Isolation	Clean-up	Type of GC	Reference
LLE Freon 11	–	1DGC	Augustyn et al., 1982b
Distillation	SPE SCX	1DGC	Lacey et al., 1991
LLE ether/hexane	–	1DGC	Kotseridis et al., 1998a, 1998b, 1999a
Distillation	SPE SCX	1DGC	Hashizume and Umeda, 1996
HS-SPME	–		Sala et al., 2000, 2002
Vacuum distillation	SPE SCX	1DGC	Roujou-de-Boubee et al., 2000, 2002
HS-SPME	–	1DGC	Hartmann et al., 2002
HS-SPME		1DGC	Panighel et al., 2010
SPE C18 cartridge,	–	1DGC	Preston et al., 2008
HS-SPME		2DGC	Ryan et al., 2005
HS-SPME		2DGC	Ryona et al., 2008
HS-SPME	–	1DGC	Pickering et al., 2008c
HS-SPME	–	1DGC	Godelmann et al., 2007
HS-SPME	HS-SPME	1DGC	Sala et al., 2004, 2005
SPE	–	2DGC	Cullere et al., 2009
–	SPE SCX	1DGC	López et al., 2011
–	HPLC	2DGC	Schmarr et al., 2010
HS-SPME	–	1DGC	Boutou and Chatonnet, 2007
Demixing/ Microextraction	–	1DGC	Ferreira et al., 2000
HS-SPME	–	1DGC	Proteau et al., 2004

Sample Preparation Procedures

To take advantage of powerful analytical techniques, appropriate sample preparation is necessary. These methods include isolation of the volatile fraction using LLE, distillation, HS-SPME, and clean-up procedures such as SPE and HPLC (see Table 2) to be used prior to analysis by GC/MS, GC/NPD, LC/MS/MS, and other methods. Recoveries of MPs from reported methods ranged from 5%–109% (Table 3).

Isolation

Liquid-Liquid Extraction

LLE is the simplest technique used most frequently for analyzing aromas, and therefore has also been adopted to quantify MPs (Slingsby et al., 1980; Augustyn and Rapp, 1982a; Augustyn et al., 1982b; Boison and Tomlinson, 1990; Kotseridis

Table 3 Recovery percentages reported in reviewed methods of MP analysis

Compound	Recovery	Reference
MP, MEMP	31–36	Sala et al., 2002
	43–58	Sala et al., 2000
ETMP	43–58	Sala et al., 2000
	78–105	Sala et al., 2002
SBMP	78–105	Sala et al., 2002
	86–103	Hashizume and Umeda, 1996
	99–102	Kotseridis et al., 2008
	94–109	Sala et al., 2000
IPMP	14	Heymann et al., 1986
	89.5–93.3	Belancic and Agosin, 2007
	78–105	Sala et al., 2002
	86–103	Hashizume and Umeda, 1996
	99–102	Kotseridis et al., 2008
	94–109	Sala et al., 2000
IBMP	5.0–10.0	Harris et al., 1987
	53	Heymann et al., 1986
	90	Kotseridis et al., 1999a
	93.4–97.4	Belancic and Agosin, 2007
	78–105	Sala et al., 2002
	86–103	Hashizume and Umeda, 1996
	99–102	Kotseridis et al., 2008
	94–109	Sala et al., 2000

et al., 1998a, 1998b, 1999b). Given that MPs are typically present in low concentrations in wine, this technique requires high MP concentrations to counter interference caused by other aromatic compounds in the wine matrix. To use this technique successfully, separation techniques are used to clean the extract relative to the sensitivity of the detector being used. The solvent used in the process can have an effect on the success of MP extraction. Trichlorofluoromethane (Freon 11) is no longer used much in MP analysis in grapes, musts, and wines, but has been used extensively for analyzing volatile compounds in foods. The usage of Freon 11 in early work with MPs in wine has been reported by several authors, where it was involved in detecting ETMP, IPMP, and IBMP in Sauvignon Blanc grapes (Augustyn and Rapp, 1982a; Augustyn et al., 1982b). Later, Freon 11 was used to extracting and concentrating IBMP in wines using low temperature and high vacuum extraction conditions (Boison and Tomlinson, 1990). The authors obtained results of 500 ng/L of IBMP in Bordeaux wines, which is an extremely high level and suggests an error in the procedure. Dichloromethane (DCM) was used by Slingsby et al. (1980) to extract MPs from a large volume of wine using twelve consecutive extractions; their first attempt extracted and identified IBMP in Cabernet Sauvignon containing a pungent herbaceous odor. Similarly, DCM was compared against several solvents including diethyl-ether, DCM/pentane, and diethyl-ether/hexane to identify which solvent allowed best extraction while achieving good recovery (Kotseridis et al., 1998a, 1999b); DCM and diethyl-ether achieved the best recoveries.

Diethyl-ether has been used in a method involving isotopic dilution and deuterated-IBMP as an internal standard by successively extracting MPs followed by a concentration step involving N_2 stream (Kotseridis et al., 1998b). Diethyl-ether/hexane used at a ratio of one volume per volume allows for reduced interference by other compounds in sample extracts while maintaining low levels of emulsion. The authors determined two simple methods for IBMP isolation and concentration involving diethyl-ether/hexane: the first method uses MEMP, while the second uses deuterated-IBMP as internal standards (Kotseridis et al., 1998a, 1999b).

LLE (DCM) could also be used as the second step after isolation by distillation, which provides high extraction efficiencies from the aqueous alcoholic phase (i.e., distillates of wine samples) (Alberts et al., 2009).

Distillation

Several researchers used vacuum/atmospheric pressure distillation or steam distillation as the first step in the sample preparation procedure to isolate the MP volatile fraction from the grapes, musts, or wines. The fact that the nonvolatile components were eliminated in this step contributed positively to the overall method and generally in obtaining a "clean" extract, also avoiding any deposits on the following chromatographic system. Subsequently, liquid extraction of the distillates, or cation exchange resins, are used as clean-up methods (Lacey et al., 1991; Allen et al., 1994; Hashizume and Samuta, 1999; Roujou-de-Boubée et al., 2000; Alberts et al., 2009).

Headspace Solid-Phase Microextraction

HS-SPME is a rapid automated technique involving no solvent that is used to quantify numerous volatile compounds in food and wine (Chapman et al., 2004b; Kataoka et al., 2000; Vas et al., 1998; Whitton and Zoecklein, 2000). This technique has been applied to analyzing aroma compounds such as MPs in wines. Numerous fibers have been used in methods involving HS-SPME for MP analysis including polyacrylate (PA), polydimethylsiloxane (PDMS), polydimethylsiloxane-divinylbenzene (PDMS-DVB), carboxen-polydimethylsiloxane (CAR-PDMS), and carbowax-divinylbenzene (CW-DVB) (Sala et al., 2000; Hartmann et al., 2002). Applications of various fibers for MP analysis are summarized in Table 4. The efficacies of these fibers were tested and the authors concluded that PDMS-DVB performs best in aqueous solution, while CW-DVB provides best results for 12% ethanol model solutions (Sala et al., 2000; Hartmann et al., 2002). Ethanol solutions are models of real wine, which is a complex solution in which ethanol competes with MPs for the fiber, often yielding low recoveries (Hartmann et al., 2002). This further displays how difficult it is to study compounds at such low concentrations, such as MPs in complex solutions containing interfering compounds. Since ethanol interacts with the fiber, a primary clean-up step involving distillation of the sample is necessary to lower ethanol content to prevent it from interacting with the fibers and skewing results of MP analysis; when analyzing grape or must samples, this pre-treatment is is unnecessary. The methodology

Table 4 Type of fibers used in reviewed methods for MP analysis

Fiber	References
PDMS	Ryan et al., 2005
	Boutou and Chatonnet, 2007
	Hartmann et al., 2002
CW/DVB	Ryan et al., 2005
	Godelmann et al., 2007
	Hartmann et al., 2002
PDMS/DVB	Ryan et al., 2005
	Sala et al., 2002
	Ryan et al., 2005
	Godelmann et al., 2007
	Sala et al., 2004
	Sala et al., 2005
	Proteau et al., 2004
	Belancic and Agosin, 2007
	Hartmann et al., 2002
PA	Hartmann et al., 2002
	Ryan et al., 2005
	Boutou and Chatonnet, 2007
DVB/CAR/PDMS	Pickering et al., 2008b
	Pickering et al., 2008c
	Ryan et al., 2005
	Ryona et al., 2008
	Boutou and Chatonnet, 2007
	Blake et al., 2009
	Cai et al., 2007
	Kotseridis et al., 2008
	Hartmann et al., 2002
	Panighel et al., 2010
CAR/PDMS	Godelmann et al., 2007
PDMS/AC	Godelmann et al., 2007

developed by Sala et al. (2000, 2002) takes advantage of MPs' decreased volatility and protonation at an acidic pH before distillation. Other authors simply dilute the wine two- or threefold to reduce the percentage of ethanol (Kotseridis et al., 2008). Alternatively, Hartmann et al. (2002) developed an HS-SPME method for analyzing IBMP levels in model wine solutions that requires minimal sample preparation; however, the detection limit was roughly 100 ng/L, which is much higher than the detection threshold of 2 ng/L in water.

Modern methods for MP analysis have been developed to enable easier sample preparation using devices such as the SPME autosampler. Chapman and colleagues (2004b) developed a method that used an SPME autosampler to decrease time consumption due to sample preparation. Different fibers and sampling temperatures were tested and they determined that PDMS/DVB/CAR fibers resulted in a two- to threefold better sensitivity. The temperatures that provided best response during sampling ranged from 40–45°C. These values agree with other reported temperatures that range from 27–45°C (Hartmann et al., 2002; Proteau et al., 2004). Earlier methods involved distillation, separation with an ion exchange column, and concentration, which took approximately two days per sample (Harris et al., 1987; Allen et al., 1991; Hashizume and Umeda, 1996). Recent methodological developments have reduced the sampling time to one hour per sample extraction; in comparison, the method developed by Sala et al. (2000) took roughly 4 hours per sample (Kotseridis et al., 2008). Hartmann et al. (2002) reported the shortest extraction time for HS-SPME of 30 minutes per sample, which was also used by Panighel et al. (2010), but such a short extraction may not provide accurate results for quantification of MPs. It is important to note that SPME is not a specific method for analyzing MPs and generally lacks isolation selectivity.

Domínguez and Agosin (2010) used a method described by Belancic and Agosin (2007) and coupled HS-SPME and GC-MS to identify IBMP in Carménère wines. They quantified IBMP concentrations at an average of 3.6 ng/L across the samples tested, which is much lower than previously reported in literature data for 2003 Carménère wines, and suggests better MP management (Belancic and Agosin, 2007). These studies demonstrate that HS-SPME is a useful isolation and concentration technique for analyzing MPs and general aromatic compounds in the food and wine industry.

Clean-Up Procedures

Solid-Phase Extraction

SPE is used independently or in combination with LLE. Five different phases are involved in five different SPE extraction modes including silica, octadecyl cartridges (C-18) and strong cation exchange resins (SCX), and more recently LiChrolut EN and Bond Elut Plexa PCX, which is a cation exchange mixed-mode sorbent.

Silica was used in early methods of determining MPs in Cabernet Sauvignon grapes in a method involving vacuum-distilled crushed berries, followed by a LLE of the distillate with the solvent pentane. Afterward, the authors used silica to perform solid-phase extraction prior to concentration and quantification (Bayonove and Cordonnier, 1971; Bayonove et al., 1975); this method involved time-consuming sample preparations.

More targeted isolation procedures using SCX resins were developed (Harris et al., 1987). The key isolation step of all these strategies was the selective retention of the MPs on an SCX resin, but the methods required wine distillation, several pH adjustments, and several liquid–liquid or liquid–solid partitions, which made the analyses extremely laborious and time-consuming. As an example, benzenesulfonic acid is a SCX resin that allows analysts to take advantage of volatility and basicity of MPs in a method involving the use of this strongly acidic resin capable of extracting MPs from the distillate. Early work for analyzing MPs involved distillation prior to extraction with a SCX resin, which was followed by elution with an alkaline buffer solution. Afterwards, LLE was performed prior to quantification using GC-MS (Harris et al., 1987; Allen et al., 1991, 1994). Alternatively, this process enables the analyst to concentrate MPs to the extent that they can be determined below their sensory thresholds from a small sample of wine ranging from 200–300 mL. The authors successfully identified and quantified IBMP, IPMP and SBMP in Sauvignon Blanc wines using this method, but there are disadvantages; for example, the recovery

rates were very low, ranging from 10 to 15%. In addition, the method involved a primary clean-up step to avoid resin saturation, and involved complicated sample preparation (Harris et al., 1987). The method was improved by using higher amounts of SCX for extractions, which increased the recovery (Hashizume and Umeda, 1996; Roujou-de-Boubée et al., 2000). Roujou-de-Boubée et al. (2002) further simplified this methodology by using steam distillation to increase the sensitivity.

Other resins have been used for MP analysis such as Porapak-Q resin beds, which were studied by Ortega et al. (2001) to see what level of thickness allowed for best quantitative recovery of MPs in wine. The authors concluded that 5 cm Porapak-Q resin beds allow limits of detections as low as 4 ng/L for IPMP and 6 ng/L for IBMP using GC-MS in a single isolation step.

Replacing distillation and SCX steps with usage of a C-18 (octadecyl) cartridge has reduced preparation time (Pickering et al., 2005). C-18 cartridges have enabled analysts to eliminate tedious steps involved in analyzing MPs such as distillation pretreatments and SCX steps (Pickering et al., 2006). The first use of C-18 cartridges for MP analysis was documented by Heymann and associates (1986) to analyze levels of IBMP and IPMP in wines; however, at this point the method involved tedious distillation and concentration steps. The authors distilled wine samples at pH 5–6 and collected the distillate in an acidic solution. This solution was then used for extraction with a C-18 cartridge, but due to the high detection limit, the procedure was applied only to spiked, synthetic wines. The wine matrix is exceptionally complex and contains numerous interfering compounds such as volatile phenolics that interfere at the detection limit of micrograms per liter. The main advantage of distillation is that it allows analysts to remove less volatile, interfering compounds from the samples but this advantage does not outweigh the time required by the process. Recent methods involving C-18 cartridges are less time consuming, and have much better detection limits that yield accurate analysis of MPs (Pickering et al., 2005). Similarly, LiChrolut EN resins and analyte elution with DCM were used by Cullere et al. (2009) using multi-dimensional gas chromatography–mass spectrometry (MDGC-MS).

SCX resins are partially selective toward MPs based on their charge state. Researchers realized that the key property of MPs is their ability to form a cation at acid pHs, thus permitting the separation of MPs from other aroma matrix compounds, and this acts as a clean-up procedure. Although, as previously mentioned, the procedures are complex and laborious and it was also mentioned that the recoveries are nonquantitative and often matrix-dependent. Thus, Schmarr et al. (2010), based on the work of the Ferreira group, proposed the use of a mixed-mode polymeric cation-exchange sorbent with SPE after the protonation as a solution to this problem. Subsequently, López et al. (2011) used mixed mode sorbents combining hydrophobic and cation-exchange extraction properties in order to achieve a simple way to concentrate, isolate and elute MPs in a single separation device. Indeed, they were able to quantify the three most important MPs at ng/L levels using Bond Elut Plexa PCX cartridges.

Overall, the usage of different modes of SPE has lead to development of reliable methods that allow very low detection limits to yield highly accurate results. Enabling analysts to use such methods provides researchers with a workable time frame as recent methods have developed to be less time consuming.

HPLC Clean-up Procedure

Recently, Schmarr and co-workers (2010) proposed a sample clean-up using solid phase extraction with a mixed-mode polymeric cation-exchange sorbent procedure followed by on-line coupled HPLC with multi-dimensional gas chromatography and detection with mass spectrometry (on-line LC–MDGC–MS). This method was choiced to avoid any interferences that the authors found in one sample, but it is a heavy procedure and not easily adopted by other analytical labs. Also recently, López et al. (2011) using the same type of SCX resin (Bond Plexa PCX) were able to quantify MPs simply using mono-dimensional GC coupled to an ion trap mass spectrometer which seems to be a very promising method. This recently published method shows to have less interferences in comparison to other methods and the fact that they were able to quantify by using mono-dimensional GC-MS makes the method more attractive as most of the analytical laboratories around the world have mono-dimensional GC-MS. These are the main differences with the method published previously by the same group, in which they used a two-dimension GC-MS method to analyze MPs (Cullere et al., 2009). It would be interesting to compare in the future the results found by this SPE-LC-MS method with other 1D or 2D GC-MS on the same samples.

CHROMATOGRAPHIC CONDITIONS

Internal Standards

Analyzing compounds based on their aromaticity can be difficult if analysts are unsure which compound is producing a specific smell. Internal standards are used as a control for analysts to know whether the desirable compound is present. There are different compounds that may be used for internal standards of MPs, which includes analogs such as isotopic labeled molecules or other pyrazines not present in grape and wines. The various internal standards are classified as alkylpyrazines, 3-alkyl-2-alcoxypyraxines, and isotope-labeled analogs of MPs (Table 5). The isotope labeled compound are usually the compound of choice but are not always commercially available which imply the need to synthesize pure compounds.

Hashizume and Umeda (1996) used 2-methyl-3-*n*-propylpyrazine instead of isotopically labeled compounds, but the results obtained were probably inaccurate as it is not chemically identical to the MPs. Tetramethylpyrazine (TMP) is another nonisotopically labeled internal standard that has been used, but it has low stability under steam distillation conditions (Heymann et al., 1986). Alternatively, others have used

Table 5 Internal standards used in reviewed methods for MP analysis

Internal standard	Reference
Acetophenone	Boison and Tomlinson, 1990
3-[1,1-$^2H_2$]-isobutyl-methoxypyrazine	Kotseridis et al., 1998b
	Kotseridis et al., 1999a
	Ryona et al., 2008
	Godelmann et al., 2007
	Proteau et al., 2004
3-methyl-2-methoxypyrazine	Kotseridis et al., 1998a
Tetramethylpyrazine	Heymann et al., 1986
3-isobutyl-2-($^2H_3$)-methoxypyrazine	Chapman et al., 2004b
	Harris et al., 1987
	Lacey et al., 1991
	Roujou-de-Boubee et al., 2000
	Ryan et al., 2005
	Allen et al., 1991
	Roujou-de-Boubee et al., 2002
	Kotseridis et al., 2008
	Blake et al., 2009
	Panighel et al., 2010
3-isopropyl-2-($^2H_3$)-methoxypyrazine	Lacey et al., 1991
	Pickering et al., 2008b
	Allen et al., 1991
	Pickering et al., 2008c
	Blake et al., 2009
	Kotseridis et al., 2008
	Panighel et al., 2010
3-secbutyl-2-($^2H_3$)-methoxypyrazine	Blake et al., 2009
	Kotseridis et al., 2008
	Panighel et al., 2010
2-methyl-3-n-propyl-pyrazine	Hashizume and Umeda, 1996
3-isopropyl-2-ethoxypyrazine	Sala et al., 2000
	Sala et al., 2002
3-ethyl-2-ethoxypyrazine	Hartmann et al., 2002
3-isopropyl-2-ethoxypyrazine	Hartmann et al., 2002
	Sala et al., 2005
	Sala et al., 2004

internal standards that are nearly identical chemically, such as 3-methyl-2-methoxypyrazine, 3-ethyl-2-ethoxypyrazine, and 3-isopropyl-2-ethoxypyrazine (Kotseridis et al., 1998b; Sala et al., 2000, 2002; Hartmann et al., 2002).

The usage of isotopically labeled analogs has increased the reliability of results from MP analysis because the internal standard and target compound are almost identical, which is advantageous for analysts as it provides a simple and accurate experimental control. The use of bi- and tri-deuterated analogs prevents coeluting compounds from interference with the natural isotopic compounds in the sample. This is particularly necessary when the isolation method leads to incomplete extraction, but may be ignored when the extraction is 100%, as it is in the case of the cation exchange clean-up methods. MS detection can differentiate between the target compound and the internal standard. In the first reliable method for quantifying MPs, Harris et al. (1987) used 3-isobutyl-2-[$^2H_3$]-methoxypyrazine as the internal standard and determined that it elutes 2–3 seconds before IBMP. The same internal standard was further integrated in different techniques; however, the use of isotopically labeled compounds as internal standards does not prevent interference—it is only by using adequate sample clean-up methods, or multidimensional gas chromatography that this could be achieved (Schmarr et al., 2010; Ryona et al., 2009).

Columns

Polar columns were generally used for MP analysis during the ‘80s and ‘90s, as well as nonpolar poly (5%-diphenyl/95% dimethylsiloxane) columns (Sala et al., 2008a). Hartmann (2003) compared the efficiencies of different polarities in columns and found that the highest resolution column was a nonpolar column (5% phenyl-dimethylpolysiloxane), which provided better results for this analysis; however, interfering compounds could be present both when using polar or nonpolar columns. In grape and must MP measurements, the IPMP peak coeluted with two compounds when using both a nonpolar and a polar column and these interferences are resolved by using two-dimensional gas chromatography (Ryona et al., 2009). No significant coelutions were observed for IBMP when using a nonpolar column.

Mono vs Multi-Dimensional Chromatography

Until recently, clean-up procedures were attempted prior to injection to the GC-MS apparatus. MDGC, two-dimensional gas chromatography coupled to nitrogen phosphorus detection (GC × GC-NPD) or to time-of-flight mass spectrometry (GC × GC-TOF-MS) was used to simplify the preparation procedures, and to avoid interference (Ryan et al., 2005). Ryona et al. (2009) correlated MP concentrations in grapes to wine with a method involving the use of two-dimensional GC analysis combined with time-of-flight mass spectrometry (TOF-MS) to detect extremely accurate concentrations in the berries. The development of their new method came with the advantages that it was a quick and simple process unlike others used in the past. This simple and rapid process has since been used successfully by others (Cullere et al., 2009; Schmarr et al., 2010; Ochiai and Sasamoto, 2011).

Alberts et al. (2009) used an LC-MS/MS method to determine MPs using reversed-phase liquid chromatography coupled with atmospheric pressure chemical ionization. The limits of detection and quantification (0.03 and 0.10 ng/L, respectively) for all three analytes make this method more sensitive than gas chromatographic methods.

Instrumental Detectors

The choice of instrumental detector depends on the medium being studied. An analyst may use a different detector when measuring levels of MPs in grapes or in wines (Table 6).

Early methods for analyzing MPs involved techniques that were unreliable because detectors were incapable of

Table 6 Instrumental parameters used in the reviewed GC methods for MP analysis by authors

Column	Detector	Clean up	Analytes	Reference
CW 20 M (23 m, 0.25 mm i.d.)	MSD	LLE Freon 11	Flavor compounds	Augustyn et al., 1982b
DB-WAX (50 m, 0.25 mm i.d.)	FID, NPD, MSD (EI, CI)	LLE Freon 11	Flavor compounds	Boison and Tomlinson, 1990
CW 20 M (50 m, 0.25 mm i.d., 0.2 μm)	MSD (EI)	LLE ether/hexane	IBMP, C-6-alcohols, β-damascenone, α- and β-ionone, free terpenols	Kotseridis et al., 1998a
CW 20 M (50 m, 0.25 mm i.d., 0.2 μm)	MSD (EI)	LLE ether or ether/hexane	IBMP, α- and β-ionone, β-damascenone	Kotseridis et al., 1998b, 1999a
DB-WAX (60 m, 0.32 mm i.d., 0.5 μm)	MSD (EI)	SPE SCX	IPMP, SBMP, IBMP	Hashizume and Umeda, 1996
CP-WAX 57CB (50 m, 0.25 mm i.d., 0.2 μm)	NPD	HS-SPME	MP, MEMP, ETMP, IPMP, SBMP, IBMP	Sala et al., 2000, 2002
BP 20 (50 m $\times$ 0.22 mm i.d, 0.2 μm)	MSD (CI)	SPE SCX	IBMP	Roujou-de-Boubee et al., 2000, 2002
HP-5MS (30 m, 0.25 mm i.d., 0.25 μm)	NPD	HS-SPME	ETMP, IPMP, SBMP, IPMP	Hartmann et al., 2002
HP-5MS (30 m, 0.25 mm i.d., 0.25 μm)	MSD (ion trap)	HS-SPME	ETMP, IPMP, SBMP, IPMP	Panighel et al., 2010
SPB-35 (30 m, 0.25 mm i.d., 0.25 μm)	NPD	HS-SPME	MP, MEMP, ETMP, IPMP, SBMP, IBMP	Sala et al., 2000, 2002
SE-54 (30 m, 0.25 mm i.d)	FID, NPD, MSD (EI, CI)	LLE freon 11	Flavor compounds	Boison and Tomlinson, 1990
SE-30 glass capillary, 60 m	MSD	LLE DCM	Flavor compounds	Slingsby et al., 1980
DB-5 (30 m $\times$ 0.25 mm i.d $\times$ 0.25 μm)	MSD	SPE C18 cartridge, LLE DCM	IBMP, IPMP	Preston et al., 2008
BPX5 (30 m $\times$ 0.25 mm i.d. $\times$ 0.25 μm)	GC-NPD, GC-TOFMS		IBMP, SBMP	Ryan et al., 2005
VF-WAXms (2.5 m $\times$ 0.10 mm $\times$ 0.1 μm); RTX5 (30 m $\times$ 0.25 mm $\times$ 0.5 μm)	GC-TOFMS		IBMP	Ryona et al., 2008
HP-5 (30 m $\times$ 0.25 mm i.d. $\times$ 0.25 μm)	MSD	HS-SPME	IPMP	Pickering et al., 2008c
VF-1701 ms (29.5 m $\times$ 0.25 mm i.d. $\times$ 0.25 μm)	MSD	HS-SPME	SBMP, IPMP, IBMP, MEP	Godelmann et al., 2007
CP-WAX 57CB (50 m, 0.25 mm i.d., 0.2 μm)	NPD	HS-SPME	IPMP, IBMP, SBMP, MEP	Sala et al., 2004, 2005
VF-5 MS (30 m $\times$ 0.25 mm i.d. $\times$ 0.25 μm)	MSD (EI), SIM	HS-SPME	Defective volatile compounds	Boutou and Chatonnet, 2007
DB-WAX (60 m $\times$ 0.32 mm i.d. $\times$ 0.5 μm)	MSD (EI)		Flavor compounds	Ferreira et al., 2000
DB-WAX (60 m $\times$ 0.32 mm i.d.)	MSD (EI)	LLE DCM	IPMP, IBMP	Hashizume and Samuta, 1999
HP-5MS (30 m, 0.25 mm i.d., 0.25 μm)	MSD	HS-SPME	IPMP, SBMP, IBMP	Blake et al., 2009
DB5-MS (30 m $\times$ 0.25 mm i.d.)	MSD	C18 cartridge	IPMP	Pickering et al., 2007b
DB5-MS (30 m $\times$ 0.25 mm i.d.)	MSD	C-18 Cartridge	IPMP	Pickering et al., 2005
DB-WAX (30 m $\times$ 0.32 mm i.d. $\times$ 0.25); CPB20 (50 m $\times$ 0.2 mm i.d. $\times$ 0.25 μm); Ultra-1 (50 m $\times$ 0.2 mm i.d. $\times$ 0.33 μm)	MSD	LLE DCM, C18 Cartridge	IPMP, IBMP	Hashizume and Samuta, 1997
BPX5 (30 m $\times$ 0.25 mm i.d. $\times$ 0.25 μm); DBWAX-ETR (30 m $\times$ 0.25 mm i.d. $\times$ 0.25 μm)	NPD, MSD (EI, ion trap)	HS-SPME	IBMP	Proteau et al., 2004

identifying compounds present at low concentrations. Flame ionization detectors (FIDs) were explored early, and were not promising; FIDs were then mainly used as a complement to GC-O analysis (Bayonove et al., 1975). Mass detection (MS) was used in the first identification of IBMP in wines by Bayonove et al. (1975), but required higher concentration factors of MPs due to its low sensitivity. Others experienced similar problems using MS even when the concentration factor was high, since the detection limit of MPs was close to the detection limits of MS detectors (Augustyn et al., 1982b). This is mainly caused by a lack of sensitivity in MS due to the total ion chromatogram (TIC) mode. Later, analysts determined that selected ion monitoring (SIM) is necessary for application of MS to MP analysis (Slingsby et al., 1980; Boison and Tomlinson, 1990; Kotseridis et al., 1998a, 1998b, 1999b). Harris et al. (1987) reported the use of MS in SIM with electron ionization (EI) and chemical ionization (CI) to identify MPs successfully. This method was further modified by Hashizume and Umeda (1996) by applying EI instead of CI. Other analysts have integrated numerous detectors into their methods by combining them with GC (Table 5).

NPDs were used by analysts as they are sensitive instrumental detectors capable of detecting very low concentrations of nitrogen or phosphorous containing compounds (Hartmann et al., 2002; Sala et al., 2000, 2002; Ryan et al., 2005). Although this detector is sensitive, it requires a high concentration factor to provide high recoveries. In addition, the sensitivity is not high enough to allow direct injection of MP samples. Hence, good cleanup is necessary to avoid detection of interfering compounds if present in high enough concentrations in the wine matrix. NPDs are typically used as a complement to GC instead of FID because it allows for peak identification (Augustyn et al., 1982b). Several analysts have applied this detector in their methods for analyzing MPs (Sala et al., 2000, 2002, 2004, 2005; Hartmann et al., 2002; Proteau et al., 2004; Ryan et al., 2005).

Gas chromatography-olfactometry (GC-O) was critical in identifying IBMP and correlating it to green pepper odor, which is characteristic of Cabernet Sauvignon wines (Bayonove et al., 1975). They used a combination of GC-O with MS that allowed them to compare retention times between the standards and the sample to determine which compounds were present. This early analysis encouraged several others to become involved in this area of research (Slingsby et al., 1980; Augustyn and Rapp, 1982a; Augstyn et al., 1982b; Heymann et al., 1986); for example, the use of smell-retention times allowed Augustyn et al. (1982b) to detect ETMP, IPMP, and IBMP for the first time in Sauvignon Blanc grapes.

Recently, the GC-O method has been improved for aroma detection by Ochiai and Sasamoto (2011) by their development of a novel selectable one-dimensional or two-dimensional gas chromatography-olfactometry/mass spectrometry with preparative fraction collection (1D/2D GC–O/MS with PFC). They applied this to detection, identification and quantification of IBMP in spiked Sauvignon Blanc samples by SBSE, followed by thermal desorption (TD) along with ^{1}D/^{2}D GC–O/MS with PFC. This method was tested for spiked wine with IBMP concentrations between 5–50 ng/L, and could be even more powerful in terms of identification than previous methods as the identification of the full mass spectrum (^{2}D TIC) of off-flavor compounds, especially IBMP at ng/L level in wine. This was made possible by using SBSE-PFC enrichment with 20 injection cycles and subsequent TD–^{1}D/^{2}D GC–O/MS analysis.

FACTORS AFFECTING MP LEVELS IN GRAPES, MUSTS, AND WINES

MP Biosynthesis

Understanding the dynamics of MP biosynthesis and accumulation in grape vines can be viewed as the key to understanding MP concentrations in wines (Ryona et al., 2009). Despite the interest in factors that influence MP accumulation, or MP degradation, little work has been done in determining the biochemical pathways responsible. In 2001, a methyltransferase capable of converting 3-isobutyl-2-hydroxypyrazine to IBMP and 3-isopropyl-2-hydroxypyrazine to IPMP was isolated from Cabernet Sauvignon wine grapes (Hashizume et al., 2001). Recently, two O-methyltransferases were cloned and shown to be capable of forming MPs via O-methylation of hydroxypyrazine (HP) precursors (Dunlevy et al., 2010). The O-methyltransferases involved in MP synthesis in *Vitis vinifera* are known to be VvOMT1 and VvOMT2, which are class II plant O-methyltransferases (Dunlevy et al., 2010). In this family of enzymes, S-adenosyl-L-methionine is required to methylate the HP-based substrate. Vallarino et al. (2011) determined the catalytic activities of VvOMT1 and VvOMT2 in MP biosynthesis, and determined that the activity of VvOMT1 is more efficient. They hypothesize that because of steric hindrance in the active site of VvOMT2, formation of the transition state is limited. The functional difference is theorized to be caused by residues H272 and M182, which Vallarino et al. (2011) suggest may be important residues that could be manipulated to control the production of MPs in grapes. Interestingly, Ryona et al. (2010) suggested that the demethylation of MPs to their corresponding HPs may be related to the ratio of their concentrations during ripening. These early biochemical studies on MP production can be applied towards further research on how vineyard management parameters can have an effect on the enzyme kinetics of OMT. In vitro studies examined how VvOMT turnover rate in grape musts is affected by various factors such as pH, water concentration, and UV light exposure. Koch and colleagues (2010) determined the location of IBMP biosynthesis by grafting clusters of different varietals onto each other and monitoring IBMP translocation and accumulation. They found that although IBMP is produced by grapevine leaves, it is not translocated to the grape. Rather, the IBMP in the grape is produced in the berry itself during development.

Table 7 Viticultural parameters in relation to impact of MP accumulation in grapes

Parameter	Effect	Reference
Sunlight exposure and pruning	Cluster exposure to light was shown to reduce IBMP accumulation by 21–44% during fruit development up to preveraison but it did not increase postveraison degradation	Ryona et al., 2008
	Leaf removal prior to veraison can help in increasing fruit exposure to sunlight leading to decreased herbaceous notes in wines such as Sauvignon Blanc	Allen et al., 1988
	Less sunlight exposure resulted in wines with lower IBMP content (Note: observed from beginning of veraison, but not studies preveraison)	Sala et al., 2004
	In preveraison grapes, light exposure results in an increased accumulation of MPs (Note: observed in harvested grapes)	Hashizume and Samuta, 1999
	As bud number and yield increase, vegetative characteristics decrease, and fruity characteristics increase	Chapman et al., 2004a
	Sunlight exposure preveraison reduces MP levels at harvest	Noble et al., 1995; Marais et al., 1999; Roujou de Boubée et al., 2001; Roujou-de-Boubée et al., 2002
Temperature	Fruit of colder regions tend to have more of a vegetative odor and contain higher MPs in wines such as Cabernet Sauvignon, Sauvignon Blanc, and Merlot noir	Slingsby et al., 1980; Heymann and Noble, 1987; Allen et al., 1988, 1991; Allen, 1993; Lacey et al., 1991; Noble et al., 1995; Hashizume and Umeda, 1996; Kotseridis et al., 1999a
Ripening duration	Well ripened grapes contain lower concentrations of IBMP	Hashizume and Umeda, 1996
	Once grape ripening is complete, the MP concentrations tend to remain constant up until harvest	Kotseridis et al., 1999a
	Herbaceous or green aroma present in Merlot noir, Cabernet Sauvignon and Sauvignon Blanc wines decreases as grape maturity evolve	Roujou-de-Boubée, 1999
Humidity	High humidity levels preveraison can cause higher IBMP levels	Allen et al., 1995; Kotseridis et al., 1999a; Roujou-de-Boubée et al., 2000
Soil type	Higher vegetative wines are associated with deep, clay-rich soils that are nutrient rich and have a high water-holding capacity	Roujou-de-Boubée et al., 2000

Viticultural Aspects

There are several aspects of viticulture that ultimately have an effect on both the biochemical processes mentioned above, which lead to the formation of MPs in grapes, and the degradation of these compounds over the course of grape development. Light exposure and climate seem to have the largest effect on both production and degradation of MPs in grapes, but, other factors such as terroir, which is a unique combination of soil variables influenced by regional climate and geography, also contribute to MP levels. Viticultural parameters in relation to MP accumulation in grapes is summarized in Table 7. Table 8 summarizes MP concentration in relation to grape varietal.

Viticultural Practices, Light Exposure, and Grape Maturation

Metabolic processes in wine grapes require sunlight. Various authors have studied the effects of light exposure on MP concentrations in wines by comparing sunlight-exposed fruit and shaded fruit (Hashizume and Samuta, 1999; Sala et al., 2004; Ryona et al., 2008). It was initially believed that the effects of light exposure on MP concentrations had two primary effects: first, the authors determined that clusters that were exposed to sunlight had higher MP concentrations in immature grapes, indicating that light exposure promotes biosynthesis of MPs (Hashizume and Samuta, 1999; Sala et al., 2004); and second, they determined that MPs are sensitive to light and are subject to photodecomposition (Heymann et al., 1986; Hashizume and Samuta, 1999; Sala et al., 2004). Thus, the impact of light exposure on the quantity of MPs is a balance of the two effects. Heymann et al. (1986) observed that exposure to light significantly decreased MP concentrations in model wines. Likewise, Allen (1993) observed that by pruning leaf layers to increase sunlight exposure decreased concentration of MPs. A review by Marais (1994) reported that MP concentrations decrease more under sunlight and higher temperature conditions, and are ultimately affected by vine vigor, leaf removal, and pruning. Canopy shading of the grapes can occur, especially in more vigorous varieties or blocks, and it has been shown to lead to an increased production of vegetative aromas in Cabernet Sauvignon wines (Allen et al., 1988; Morrison and Noble, 1990; Allen, 1993). Leaf removal prior to veraison can increase fruit exposure to sunlight and reduce herbaceous notes in wines such as Sauvignon Blanc (Allen et al., 1988). The timing and severity of leaf removal determines to the lower MP concentrations; the earlier the treatment, the better the results (Allen et al., 1988; Arnold and Bledsoe, 1990). Cluster exposure to light was shown to reduce IBMP accumulation by 21–44% during fruit development up to preveraison, but it did not increase postveraison degradation (Ryona et al., 2008). Similarly, the effects of sunlight exposure preveraison reduced MP accumulation at harvest

(Noble et al., 1995; Marais et al., 1999; Roujou de Boubee et al., 2001; Roujou-de-Boubée et al., 2002). Alternatively, Sala et al. (2004) demonstrated that grape clusters shaded since veraison contained considerably less IBMP. They also determined that IBMP concentrations during ripening were similar for fruit exposed to light and for fruit covered with sackcloth. Conversely, the levels of SBMP did not change between covered- and light-exposed clusters throughout the growing season. Ultimately, Sala et al. (2004) concluded that reduced sunlight exposure resulted in wines with lower IBMP content; these results contradict the previous studies described above which showed that higher light exposure contributed to lower MP levels at harvest (Augustyn and Rapp, 1982a; Kotseridis et al., 1999a; Roujou-de-Boubée, 1999, Roujou de Boubee, 2001). Sala et al. (2004), however, measured effects of cluster shading from the beginning of veraison, not preveraison, whereas other studies report that the effect of light exposure on MP accumulation are most critical preveraison (Noble et al., 1995; Marais et al., 1999; Roujou de Boubee, 2001; Roujou-de-Boubée et al., 2002; Ryona et al., 2008). Hashizume and Samuta (1999) also reported that in preveraison grapes, light exposure results in an increased accumulation of MPs; however, this study examined harvested grapes in a glass vial exposed to artificial light rather than grapes left on the vine, which may be a confounding factor.

The type of pruning performed influences canopy density during grape development and thus affects MP levels (Allen and Lacey, 1993; Chapman et al., 2004a, 2004b). Allen and Lacey (1993) determined that minimal pruning yielded a loose and open canopy with small clusters and small-berried fruit that was more readily exposed to sunlight, which was related to lower MP levels in grapes as compared to vines that were spur pruned. In contrast, However, Sala et al. (2004) observed reduced MP levels, which suggests that leaf removal may result in effects other than changes in cluster light exposure. However, the reported MP reduction may also be due to the issue regarding the time of grape development in which the observations were made. As mentioned before, Sala et al. (2004) did not observe the effect of light exposure via leaf removal preveraison, but rather observed the effect from the beginning of veraison. The effect of growing vines on a trellis system was analyzed by Sala et al. (2004) to compare differences between trellis-trained and goblet-trained vines. The authors concluded that goblet-trained vines had significantly lower IBMP content while IBMP content in wines from trellis-trained vines was higher than the sensory threshold in red wines (Kotseridis et al., 1998a). Chapman et al. (2004a) examined the effect that bud numbers per vine had on the intensity of vegetative aroma, bitterness, and astringency in red wines and that wines from vines pruned to low bud numbers were higher in vegetative aroma and flavor, including bell pepper aroma, bitterness, and astringency relative to wines made from high yields. Meanwhile, the wines made from vines with higher bud numbers displayed higher red/blackberry notes, fruity aromas, and flavors; however, the authors reported no differences in sensory evaluation between wines made from different cluster thinning treatments. Overall, they determined that as bud number and yield increase, vegetative characteristics decrease, and fruity characteristics increase. These results suggest that aroma complexity in Cabernet Sauvignon is dependent on yield manipulation, but only if the yield is altered earlier than veraison during fruit development.

The combination of appropriate viticultural practices significantly influences MP levels in resultant wines as viticulturists decide on the type of trellising, if any, they will use. Furthermore, vine growth is manipulated through use of proper pruning techniques in an attempt to control canopy density and vine vigor, which affects sunlight exposure. To understand how MPs accumulate in grapes, it is necessary to determine how viticultural practices affect their presence to identify the ideal growing systems for controlling or diminishing MPs altogether.

To minimize MP-associated aromas in wines, the harvest date should correlate with minimum MP levels (Kotseridis et al., 1999a). Previously, Kotseridis et al. (1999a) reported that delaying harvest by 15 days can diminish IBMP levels in the grapes, and thus decrease it in the resultant wines. The effect of grape maturity on MP concentrations has been reported, in which it is reported that increased ripening duration relates to a decrease in MP content but further understanding is still needed at the molecular biochemical level (Lacey et al., 1991; Kotseridis et al., 1999a; Roujou-de-Boubée, 1999; Roujou-de-Boubée et al., 2000; Sala et al., 2004). The herbaceous or green aroma present in Merlot noir, Cabernet Sauvignon, and Sauvignon Blanc wines decreased as grape maturation is prolonged (Roujou-de-Boubée, 1999). Likewise, the sensory perception of these compounds also decreased as grape maturation proceeded (Lacey et al., 1991; Allen, 1993; Allen et al., 1995). As always, the climatic conditions will vary from season to season and the influence on grape maturity and ripening will ultimately affect the amount of MPs in the fruit at harvest.

The accumulation patterns of IBMP, IPMP, and SBMP have been studied extensively (Lacey et al., 1991; Hashizume and Samuta, 1999; Roujou-de-Boubée et al., 2000; Sala et al., 2004; Ryona et al., 2008). IBMP and IPMP both increase in concentration during early grape development (Hashizume and Samuta, 1999). IPMP levels decreased preveraison while IBMP rapidly decreased post-veraison with the major decrease occurring in the initial stages of grape maturation. Sala et al. (2004) determined that IBMP content in Cabernet Sauvignon, Merlot noir, and Sauvignon Blanc varieties decreased during grape maturity mainly during the first stage of ripening, which supports previous studies (Allen, 1993; Lacey et al., 1991; Roujou-de-Boubée et al., 2000; Sala et al., 2000).

Grape ripening plays an integral role in wine quality as it has been shown that well-ripened grapes contain lower concentrations of IBMP (Hashizume and Umeda, 1996). The accumulation patterns of SBMP and IPMP in the grape over time appear to be different than IBMP as they both tend to increase during the first stage of grape ripening followed by a decrease to roughly 11–16 ng/L, which is similar to the concentration of IBMP at

Table 8 Selected MP concentrations observed among grape varieties

Grape variety	Concentration and MP type (pg/g or ng/L)	Observed in:	Reference
Cabernet Franc	9 pg/g (IBMP)	Berry homogenate at harvest	Koch et al., 2010
	10 pg/g (IBMP)	Berry homogenate at harvest	Ryona et al., 2008
	97.4 pg/g (IBMP)	Berry homogenate pre-veraison	Ryona et al., 2008
Cabernet Sauvignon	10 ng/L (IBMP)	6 wine samples (avg)	Alberts et al., 2009
	2–24 ng/L (IBMP)	16 wine samples (range)	Preston et al., 2008
	2–16 ng/L (IPMP)	16 wine samples (range)	Preston et al., 2008
Sauvignon blanc	3 pg/g (IBMP)	Berry homogenate at harvest	Koch et al., 2010
	26.1–27.8 ng/L (IBMP)	Wine samples	Ryan et al., 2005
Semillon	2 pg/g (IBMP)	Berry homogenate at harvest	Koch et al., 2010
Chardonnay	0.53 ng/L (IBMP)	6 wine samples (avg)	Alberts et al., 2009
Pinotage	1.5 ng/L (IBMP)	6 wine samples (avg)	Alberts et al., 2009
Shiraz	2.4 ng/L (IBMP)	6 wine samples (avg)	Alberts et al., 2009
Chenin blanc	0.59 ng/L (IBMP)	4 wine samples (avg)	Alberts et al., 2009

this stage. After stage two of ripening, the concentrations of all three MPs drops to an average of below 5 ng/L at harvest (Sala et al., 2004). During ripening, temperature has a significant impact on IBMP levels in the resultant wines by observations that colder regions produce grapes with higher MP levels and more vegetative odors (Allen et al., 1988; Allen, 1993). Once grape ripening is complete, the MP concentrations tend to remain constant until harvest (Kotseridis et al., 1999a). Furthermore, IBMP levels have a strong correlation to malic acid degradation in wine grapes (Lacey et al., 1991; Roujou-de-Boubée et al., 2000).

It is clear that a better understanding of grape maturity will provide better understanding of the MP concentration at harvest and in the resultant wine. Ryona et al. (2009) demonstrated that the concentration of IBMP in resultant wines was 67 $\pm$ 13% of the levels present in the grapes. Although the literature indicates that maceration has a significant effect on MP levels in wine (Hashizume and Umeda, 1996), the exact factors that influence this transfer is unknown. The same is true for the effects of fermentation on MPs (Ryona et al., 2009). Clearly, viticultural practices remain the primary variable in determining and predicting MP levels in wine. Delaying the harvest can lower IBMP levels in the fruit, thus it is important for wine regions that exhibit higher herbaceous or green odor characteristics in wines to select appropriate harvest dates in an attempt to minimize MP levels prior to vinification, if the weather permits.

Climate

Climatic conditions are important for achieving high-quality wines, and in the case of reds, high color and sufficient tannin content. Wines from cooler regions, such as Cabernet Sauvignon, Sauvignon Blanc, and Merlot noir wines, tend to have more of a vegetative odor and contain higher MPs (Slingsby et al., 1980; Heymann and Noble, 1987; Allen et al., 1988, 1991; Allen, 1993; Lacey et al., 1991; Noble et al., 1995; Hashizume and Umeda, 1996; Kotseridis et al., 1999a). During ripening, vines in cooler regions experience greater water availability and soil fertility, which leads to increased vigor and shading, which both have influences on IBMP concentration (Allen et al., 1991, 1994; Lacey et al., 1991; Roujou-de-Boubée et al., 2000). Climatic conditions influence the reduction of IBMP levels during the final stages of ripening, which then affects MP levels at harvest (Roujou-de-Boubée, 1999; Roujou-de-Boubée et al., 2000). Temperatures during ripening have been linked to a more significant impact on IBMP levels than sugar accumulation, which leads to lower IBMP concentrations in warmer years than cooler years when compared to sugar accumulation at certain stages (Allen et al., 1991, 1994).

High humidity levels preveraison can cause higher IBMP levels (Allen et al., 1995; Kotseridis et al., 1999a; Roujou-de-Boubée et al., 2000). Grapes infected with *Botrytis cinerea* also produce wines with high IBMP levels, which may be because this fungus often proliferates in regions with higher humidity (Kotseridis et al., 1999a). The suggested mechanism for the increase in IBMP levels is that the fungus shrinks the grapes, which makes the skins more delicate and the MPs easily extracted during maceration. Clearly, an understanding of the influence of climatic conditions on MP accumulation in grapes is necessary to predict annual MP content.

Soil

Soil characteristics influence MP content in wines since soil drainage, fertility, and water-holding capacity influence vine vigor (Noble et al., 1995; Roujou-de-Boubée et al., 2000). Certain soil types with higher or lower vegetative odorants in wines, for example, wines with stronger vegetative flavors/aromas are associated with deep, clay-rich soils that are nutrient rich and have a high water-holding capacity (Roujou-de-Boubée et al., 2000). In contrast, fruitier wines were associated with shallow, sandy soils that are nutrient deficient with low water-holding capacities (Noble et al., 1995). Sala et al. (2005) analyzed the effects of irrigation and plantation density on MP content in grapes and successfully identified and quantified IPMP, IBMP, and SBMP in all samples. Irrigated vines produced higher average concentrations of IBMP relative to nonirrigated vines. These results are consistent with those from Chapman et al. (2005), in which it was determined that wines from vines with minimal irrigation had significantly lower vegetal aroma character than wines produced from vines with standard irrigation. MP levels were higher on average in wines made from blocks with a

higher plantation density. These results are significant because modern cultivation practices in many wine regions have adopted high-density plantations to maximize yield.

Multi-Colored Asian Lady Beetles (Harmonia Axyridis)

Harmonia axyridis, commonly known as the multi-colored Asian lady beetle (MALB), is a vineyard pest found in many wine regions throughout the world including Europe, North America, and South America (Pickering et al., 2008a). MALB causes off-odors and flavors in juices and wines, known as "lady bug taint" (LBT), when harvested with the grapes. If present during fermentation, MALB can be detrimental to wine quality by contributing peanut, herbaceous, bell pepper, and asparagus-like aromas and flavors. IPMP is excreted by MALB, which functions as an aggregation pheromone and an alerting signal to other beetles (Pickering et al., 2008a). This taint affects wine quality directly by influencing ortho-nasal aroma, retro-nasal aroma and taste, and indirectly by masking varietal and other desirable sensory attributes of white and red wine (Botezatu and Pickering, 2010). The effect is especially pronounced for ortho-nasal aroma, which has been found to be the most sensitive sense to LBT (Pickering et al., 2007b). In addition to increasing vegetal aroma and taste, high concentrations of MALB in grape juice significantly increase sourness and astringency, and reduces the honey-like and sweet flavors. A sensory panel demonstrated a significant decrease in preference of grape juices with higher MALB/L concentrations (Weekes et al., 2010). Pickering et al. (2005) analyzed common commercially available fining agents to identify a treatment for LBT. Activated charcoal lowered IPMP levels in white wines, and deodorized oak chips lowered LBT in red wines. Overall, oak chip treatment reduced intensity of LBT in the white and red wines sampled; however, the aroma characteristic exhibited by LBT remains stable after bottling and will not decrease while aging occurs. LBT-causing IPMP has a low detection threshold ranging from 0.32–2.29 ng/L in wine (Pickering et al., 2007a), thus, controlling proliferation of these beetles is extremely important as they can contribute strong off-odors and flavors that negatively affect wine quality.

Pickering et al. (2007b) determined that 200–400 beetles per ton of grapes is sufficient to cause LBT in wines. To determine whether dead MALB cause LBT if present during fermentation, they produced wine from juice with added MALB that had been dead for 1, 3, 7, and 60 days to juice, and concluded that IPMP concentration was considerably higher in wine produced from live beetles. They further suggested that extended maceration may lead to higher extraction levels of IPMP from MALB. SBMP content in wine fermented with live MALB increased along with IPMP, while IBMP concentrations remained unchanged (Kotseridis et al., 2008). MALB that were dead for one day before addition to juice increased SBMP, however, MALB that had been dead for three days or longer did not. Kotseridis et al. (2008) clarified the juice before fermentation and obtained lower IPMP in the resultant wine relative to wines produced from unclarified juices. Cai et al. (2007) analyzed live MALB to identify and quantify four different MPs: 2,5-dimethyl-3-methoxypyrazine, IPMP, SBMP, and IBMP (Cai et al., 2007). They estimated the detection limits of IBMP, SBMP, and IPMP to range between 0.020–0.022 ng/L. The results suggest that IPMP and SBMP are not the only MPs that contribute to LBT, but they are the most abundant.

In general, it is best to control populations of MALB well before harvest to avoid contamination of the juice with live and dead lady bugs, and avoid the resultant off-odors and flavors associated with "lady bug tainted" wines (Pickering et al., 2008a). In instances where MALB infestations do occur, it is possible to add commercial fining agents, such as activated charcoal for white wines, and deodorized oak chips for red wines, to reduce LBT and produce a quality wine (Pickering et al., 2005).

Winemaking

Vinification Stage

At the end of the growing season, the grapes are placed in the winemaker's hands for the winemaking process. Methods developed by Ryona et al. (2009) allow enologists to measure the MP content in grape berries before harvest to predict MP concentrations in the resultant wine (Kotseridis et al., 1999b, Ryona et al., 2009). It was found that MP concentrations in the resultant wines are 67 $\pm$ 13% of the levels in the corresponding berries at harvest. Since the skins, pulp, and stems contain higher MP levels than their corresponding juices (Hashizume and Samuta, 1996; Hashizume and Umeda, 1996; Kotseridis et al., 1999b; Roujou-de-Boubée et al., 2002), it is important that proper destemming procedures are followed to avoid stem presence during vinification as they can contribute to an increase in IPMP, SBMP, and particularly IBMP in final wines (Hashizume and Umeda, 1996; Roujou-de-Boubée et al., 2002). The mode of extraction of MPs from fruit to juice was believed to be due to ethanol extraction and from precursors released by certain yeast strains (Marais, 1994; Roujou-de-Boubée et al., 2000). IBMP levels increased in the first twenty-four hours of maceration prior to fermentation, which suggests that neither ethanol nor certain yeast strains are responsible for extraction into juice (Allen et al., 1994; Marais, 1994). The concentrations of SBMP and IPMP did not increase during vinification suggesting that perhaps the skins contain lower concentrations of IPMP and SBMP relative to IBMP (Sala et al., 2004). The concentrations of MPs in juices after crushing may actually be two to three times lower than the freshly fermented wine (Allen et al., 1994, 1995; Hashizume and Umeda, 1996; Sala et al., 2000, 2002). The transfer of MPs successively from the fruit and ultimately to the wine appears to be primarily dependent on maceration times (Hashizume and Umeda, 1996). Sala et al. (2004) determined that MP concentrations increased after one day of maceration, and no further increases were observed after racking. They also examined the effects of malolactic fermentation (MLF) after

alcoholic fermentation and concluded that MLF had no effect on MP levels in the wine.

Enologists must use available techniques to minimize MP levels in many regions where wines have problematic MP levels. Choosing appropriate maceration times to allow extraction of necessary compounds without risking overextraction of MPs is an issue for the winemaker. Clarification is a fine method to reduce MP levels in resultant wines, particularly in white wines (Roujou-de-Boubee et al., 2002; Kotseridis et al., 2008). Thermo-vinification is a the process of heating juice to 60–80°C for a short period prior to fermentation to increase phenolic extraction, which can reduce IBMP content in red wine from 29 to 67% (Roujou de Boubee et al., 2004). Another possible treatment for wines is the use of polysaccharides, such as yeast-derived mannoprotein products, to minimize herbaceous and vegetal aromas and flavors; however, while there is some anecdotal evidence, no studies have demonstrated the ability of these polysaccharides to "bond" with pyrazines, and it may simply be that pyrazine aromas are "buried" within other odorant compounds. Another anecdote suggests that micro-oxygenation reduces the vegetal character of the wines. All these methods do not select for MPs, and thus influence the concentrations of other odorant compounds or other wine parameters. In 2010, a patent was given to a method that uses a protein to eliminate MPs (Inglis et al., 2010); a protein is added to the grape or juice that binds to MPs at a pH of about 3–4, which then forms a protein-MP complex, and subsequently removes the protein-MP complex from the grape or grape product. This method was found to effective for reduction of MP levels, reducing up to 60% of MPs. The patent also describes method in which MPs are removed from samples by contacting the sample with a polyethersulfone membrane at conditions that facilitate MP removal—this ultimately allows the polyethersulfone membrane to act as a filter for reducing MP content.

The methods may be used to remediate high levels of MP in wine; unfortunately, these are not always appropriate or feasible, because they may have a further negative effect on wine quality, or the wine that is produced may not be up to the standard originally intended by the winemaker.

Ageing

Certain factors such as bottle color, bottle versus tetrapak, and corks versus screwtops in packaging were examined to determine whether packaging and storage methods affect MP levels in wines (Maga, 1990; Blake et al., 2009). Bottle color influenced concentrations of MPs in the wines studied: over a one-year period, MP concentrations in clear bottles exposed to light decreased up to 60% faster relative to wine in clear bottles maintained in darkness; MP concentrations decreased up to 40% faster in green or amber bottles exposed to light, relative to wine in green or amber bottles maintained in darkness. MP contents remained stable in glass bottles of any color that were stored up to one year in darkness (Maga, 1990). Blake et al. (2009) analyzed packaging and closure options and found that after an 18-month period, the MP content in tetrapak stored wines decreased by roughly 50%. The MP content in wines sealed with synthetic cork showed a greater decrease than wines sealed with screw tops and real corks. These phenomena are probably to adsorption. The authors concluded that the difference in gas permeability from different packaging options is induces changes in wine composition during aging that could affect sensory perception.

The reduction of MP concentration in wines during aging appears to be due to complexing with polyphenolic compounds present in the wine matrix; there are greater drops in IBMP concentrations in red wines, which have more phenolic compounds (Blake et al., 2009, 2010). This hypothesis is further supported by Lund et al. (2009) who found that IBMP perception in Sauvignon Blanc wine is largely suppressed as a result of the addition of polyphenolic compounds. They too hypothesize that this is due to non-covalent bonding between polyphenols and IBMP.

Microorganism and Yeast-Strain Selection

Gallois et al. (1988) and Pickering et al. (2008c) examined the effect of microorganisms and specific yeasts on MP concentrations. Gallois et al. (1988) reported that *Pseudomonas taetrolens* is capable of producing IPMP in relatively high concentrations, which can contribute vegetative odor to wines. Conversely, specific yeast strains are capable of either masking or intensifying herbaceous odors produced by MPs (Pickering et al. 2008c). The authors concluded that the yeast Lalvin BM45 caused an 11 ng/L or 29% increase in IPMP levels during fermentation, which is substantial.

CONCLUSION

MPs are nitrogenous heterocyclic compounds that are important to food science, including enology and viticulture, because of their potent contribution to vegetative and earthy aromas, in winemaking and viticulture, it is important to control the accumulation of MPs to maintain MP concentrations within desirable levels that contribute to, but do not negatively affect the character of the wine. For these reasons, MPs are an important group of compounds to study to determine the factors that affect the concentration and perception of MPs in the entire wine-making process, from grape development to bottle aging. Unfortunately, MPs are present in small amounts, and efficient extractions and powerful analytical techniques must be harnessed to detect and quantify MPs in samples. As these methods are improved, our understanding of these compound in grapes, musts, and wine can be better understood, and so too our ability to control MP accumulation.

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