Identification of *O-methyltransferase* genes involved in the biosynthesis of 3-alkyl-2-methoxypyrazines in grapevines (*Vitis vinifera* L.)

by

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Abstract

3-alkyl-2-methoxypyrazines (MPs) are a family of potent volatile aroma compounds commonly found in various vegetables. MPs, in particular 3-isobutyl-2-methoxypyrazine (IBMP), can also be found in the grapes and subsequent wines of a small number of grapevine cultivars, including Cabernet Sauvignon and Sauvignon blanc. MPs have odour perceptions commonly described as herbaceous and green capsicum-like, which when present in wines at high concentrations are considered detrimental to wine quality. Controlling the level of MPs in wines is best achieved by manipulating the amount that accumulates in the berry, which is highly variable and influenced by numerous environmental and viticultural factors. Little is known about the biosynthesis of MPs, however a previous study has shown that in grape berries an *O*-methyltransferase (OMT) enzyme catalyses the final step in MP biosynthesis via the methylation of 3-alkyl-2-hydroxypyrazine (HP) precursors (Hashizume *et al.*, 2001a). Furthermore a protein with this activity has previously been purified from the shoots of Cabernet Sauvignon and its N-terminus partially sequenced (Hashizume *et al.*, 2001b).

The aim of this project was to identify the gene encoding the OMT responsible for the final step of MP biosynthesis in grape berries. Using a candidate gene approach the gene *VvOMT1* was identified showing an exact homology to the 22 amino acid sequence of the native protein purified Hashizume et al (2001b) and a second gene *VvOMT2* found with close homology to *VvOMT1*. Kinetic analysis of recombinant VvOMT1 and VvOMT2 revealed that they each possessed methylating activity against HP substrates similar to that reported by

Hashizume et al (2001b), and the expression of *VvOMT1* was found to coincide with the period of MP biosynthesis in early grape development. However, RNAi mediated silencing of both *VvOMT1* and *VvOMT2* in grapevine hairy-roots did not result in a reduction of MPs.

A mapping approach was also used to identify genes responsible for IBMP accumulation. A population of F2 progeny derived from a cross between Cabernet Sauvignon (CS) and a Pinot Meunier dwarf mutant (PM dwarf) was established and found to segregate for the trait of IBMP accumulation in young berries. Using the online genome sequence as a basis, CAPs and dCAPS markers were designed to the genomes of CS and PM, which enabled the identification of a 2.3 Mb locus that segregates with the trait of IBMP accumulation. A search of the online annotated genomic database revealed two putative OMTs, *VvOMT3* and *VvOMT4*, located within this locus. An association mapping study directed to this locus revealed that the gene *VvOMT3* is highly associated (p = 0.005) with the trait of IBMP accumulation in 91 existing grapevine cultivars. Furthermore recombinant VvOMT3 was found to have between 500 - 5,000 fold greater catalytic activity against IBHP than other VvOMTs investigated. The expression of *VvOMT3* also coincided the period of MP accumulation in young grape berries and was associated with IBMP accumulation in a subset of the segregating F2 progeny. Finally the elimination of sunlight from grape bunches was found to significantly reduce the expression of *VvOMT3*, resulting in a reduction of IBMP levels.

Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Jake Dunlevy

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Abbreviations

BLAST	basic local alignment search tool
CAPS	cleavage amplified polymorphic sequence
cDNA	complementary DNA
CS	Cabernet Sauvignon
CSIRO	Commonwealth Scientific and Industrial Research Organisation
dCAPS	derived cleavage amplified polymorphic sequence
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediamine-*tetra*-acetic acid
EST	expressed sequence tag
g	gram(s)
g	relative centrifugal force
GFP	green fluorescent protein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HP	hydroxypyrazine
IBHP	2-isobutyl-3-hydroxypyrazine
IBMP	2-isobutyl-3-methoxypyrazine
IPHP	2-isopropyl-3-hydroxypyrazine
IPMP	2-isopropyl-3-methoxypyrazine
kb	kilobase pairs
kDa	kilo Dalton
L	litre(s)
LB	Luria broth
LD	linkage disequilibrium
M	molar
MES	2-(N-morpholino)ethanesulfonic acid
min	minute(s)
MP	methoxypyrazine
m/z	mass-to-charge ratio
OMT	*O*-methyltransferase
PAGE	polyacrylamide gel electorphoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PM	Pinot Meunier
PN	Pinot noir
PVPP	polyvinylpolyprrolidone
rpm	revolutions per minute
SBMP	2-*sec*butyl-3-methoxypyrazine
SDS	sodium dodecyl sulphate
TBE	tris-borate-EDTA
Tris	tris(hyroxymethyl)aminomethane
v/v	volume per volume
Vv	*Vitis vinifera*
wpf	weeks post flowering
w/v	weight per volume

Chapter 1 - General introduction

1.1 Grapes and Wine

For millennia high quality wine has been prized for its exceptionally pleasurable flavour and alcoholic influences. There is evidence to suggest that wine was being made in northern Iran as early as 5,400 BC (McGovern *et al.*, 1996) and in ancient Greek mythology Dionysus was revered as the God of wine (McGovern, 2004). Today, wine is still a highly valued commodity, so much so that the grapevine (*Vitis vinifera* L.) is the most economically important fruit crop in the world (Myles *et al.*, 2011; Vivier and Pretorius, 2002), with some 8 million hectares of vineyards established worldwide (Vivier and Pretorius, 2000). It has been estimated that there are over 5,000 different cultivars of *V. vinifera* grapes grown worldwide (Alleweldt, 1994). However, only a small number of elite cultivars constitute the vast majority of the global wine market.

The major cultivars of wine grapes grown in Australia are Shiraz (25.5%), Chardonnay (21.6%), Cabernet Sauvignon (13.3%), Merlot (6.8%), Muscat (5.0%), Semillon (4.5%) and Sauvignon blanc (4.4%) with other less common varieties including Pinot noir, Pinot gris, Riesling and Grenache (Source: Australian Wine and Brandy Corporation). Currently, Australia is the 9th largest wine producing country in the world (Souce: http://faostat.fao.org; Food and Agriculture Organization of the United Nations) but is ranked 4th largest in total wine exports. In the 12 year period between 1994-95 and 2006-07 the value of Australian wine exports increased yearly to go from AUD$385 million to a peak of AUD$2.9 billion in 2006-07. However, in the three years following 2006-07 the value of Australian wine exports has steadily declined to $2.2 billion in 2009-

10 (Source: Australian Bureau of Statistics). The reduction in the value of exports in recent years is not reflected in the total volume of wine exported, which in 2009-10 was greater than the volume exported in 2006-07. It is thought that the reduction in export sales is in large due to a current surplus of wine grapes grown in Australia further compounded by reduction in global demand as a result of the global financial crisis. In order for the value of Australian wine exports to remain strong there is a need to ensure that the quality of exported wine remains high or that the product suits new markets. A predominant attribute of wine that is associated with quality is its flavour and aroma.

1.2 Wine flavour and aroma

The flavour of wine, as perceived by the consumer, is determined by the complex mixture of volatile and semi-volatile compounds present. The flavour compounds of a finished wine are commonly grouped into three categories; grape-derived, fermentation-derived and post-fermentation-derived. Fermentation-derived flavour compounds may be produced by yeast or other microbes during the fermentation process from grape berry precursors in the form of secondary metabolites or from more extensive metabolism of grape primary metabolites such as sugars and amino acids. Therefore, the type of yeast strain and/or species used during the primary alcoholic fermentation can greatly affect wine flavour, as can the strain of bacteria used during malolactic fermentation (Swiegers *et al.*, 2005). Post-fermentation treatments such as oak barreling, fining treatments and bottle-ageing also influence the complexity of wine flavour by introducing, removing or altering the array of flavour compounds. However, grape-derived aroma compounds have a significant role to play in determining wine flavour and aroma attributes. This is evident in the fact that wines produced using different grape varieties have fundamentally different flavour and aroma characteristics, which is due to the differing compositions of the berries (Dunlevy *et al.*, 2009). The ability of different grape varieties to synthesize different aroma compounds is determined by the differences in the genomes of the varieties. Nevertheless, the flavour profile of any given grape variety is also influenced by many factors, such as the environmental conditions during the growing season, management of the vineyard and harvest timing.

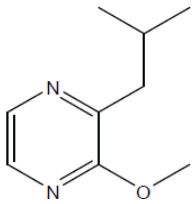
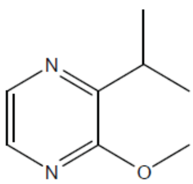
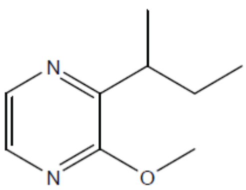
Sensory attributes that are prominent and easily distinguishable in certain varieties are known as varietal characters. Examples of varietal characters include the floral aromas commonly associated with Muscat varieties, which are attributed to high levels of a group of volatile compounds known as monoterpenes (Strauss *et al.*, 1986; Rapp, 1998) and the kerosene-like flavour common to aged Riesling wines. This kerosene-like character has been shown to be due to the presence of the norisoprenioid,1,1,6-trimethyl-1,2-dihydronaphthalene (TDN), which is formed via the acid hydrolysis of grape-derived carotenoids (Simpson, 1978; Winterhalter, 1991). Another prominent varietal character is the herbaceous or vegetative flavour that has long been associated with a number of grape varieties originating from the Bordeaux region, including Sauvignon blanc, Cabernet Sauvignon, Cabernet franc, Carmenere and Merlot (Belancic and Agosin, 2007; Lacey *et al.*, 1991). It has been shown that these distinct herbaceous characters are attributed to the presence of aroma compounds collectively known as methoxypyrazines (Lacey *et al.*, 1991; Noble *et al.*, 1995).

1.3 Methoxypyrazines

1.3.1 Identification and properties of methoxypyrazines

3-Alkyl-2-methoxypyrazines (MPs) are a family of volatile aroma compounds most commonly found in, and associated with, various vegetables. MPs are among some of the most potent aroma compounds known, with extremely low odour detection thresholds (Table 1.1) that have been reported as low as 1-2 $ng.L^{-1}$ in water (Abassi *et al.*, 1998; Buttery *et al.*, 1969; Murray *et al.*, 1970; Seifert *et al.*, 1970). As MPs are extremely potent they are typically found in nature in very low concentrations, which made their initial identification a challenging task.

Table 1.1 – Structures and odour properties of MPs

Name	Structure	Odour Description	Odour Threshold
3-Isobutyl-2-methoxypyrazine (IBMP)		Green capsicum[1, 2, 3] Herbaceous[4] Earthy[4] Musty[3] Leafy[3]	2 $ng.L^{-1}$ in water[1, 3] 2 $ng.L^{-1}$ in model wine[3] 1 $ng.L^{-1}$ in white wine[5] 15,16 $ng.L^{-1}$ in red wine[3, 6]
3-Isopropyl-2-methoxypyrazine (IPMP)		Green capsicum[1, 3] Green peas[2] Asparagus[8] Earthy[3, 4]	1,2 $ng.L^{-1}$ in water[3, 8] 2 $ng.L^{-1}$ in model wine[3] 2 $ng.L^{-1}$in white wine[5] 2 $ng.L^{-1}$in red wine[3]
3-sec-butyl-2-methoxypyrazine (SBMP)		Galbanum oil[2] Moth balls[7] Green peas[2] Musty[3]	1 $ng.L^{-1}$ in water[8]

[1] (Buttery *et al.*, 1969), [2] (Murray and Whitfield, 1975), [3] (Maga, 1989), [4] (Hashizume and Samuta, 1997), [5](Allen *et al.*, 1991), [6] (Roujou de Boubee *et al.*, 2000), [7] (Mihara *et al.*, 1990), [8] (Murray *et al.*, 1970)

3-Isobutyl-2-methoxypyrazine (IBMP) was first identified and isolated from green capsicum (bell pepper), where it was found to be the principle compound responsible for the distinct capsicum odour (Buttery *et al.*, 1969). In this study, 5 kg of capsicums were required to effectively identify IBMP by mass, infrared and UV absorption. Subsequently, the compound 3-*sec*-butyl-2-methoxypyrazine (SBMP) was identified from galbanum oil (Bramwell *et al.*, 1969), in which it is the major odorant and hence SBMP is described as having an odour reminiscent of this oil (Murray and Whitfield, 1975). The perception of SBMP has also been referred to as "moth ball-like" (Mihara *et al.*, 1990). A third MP, 3-isopropyl-2-methoxypyrazine (IPMP), was later identified in green peas (Murray *et al.*, 1970). IPMP is commonly described as "sweet pea-like" and "asparagus-like" (Murray *et al.*, 1970), but it is also sometimes associated with the earthy aroma reminiscent of roots (Gerber, 1977).

A later study utilised early gas chromatographic techniques to survey these three MP compounds in 27 common vegetables (Murray and Whitfield, 1975). This study found that at least one MP compound was detectable in all but four of the vegetables examined and all three compounds were present in 13 of the 27 vegetables. Not unexpectedly, green capsicum was found to contain the highest concentration of IBMP (~50,000 $ng.L^{-1}$), with IPMP the highest in green peas (~3,500 $ng.L^{-1}$) and the greatest amount of SBMP was found in beetroot (~5,600 $ng.L^{-1}$).

1.3.2 Biological function of methoxypyrazines

MPs are widely distributed throughout the biological kingdoms and have been identified in insects, vertebrates, marine organisms, fungi and bacteria (Gallois *et al.*, 1988; Cheng *et al.*, 1991; Gerber, 1977; Murray and Whitfield, 1975). The convergent appearance of these compounds in many unrelated species suggests that MPs have a significant biological function. A major clue as to the possible function of MPs is that they have been found to be present at high levels in many aposematic (warningly coloured) toxic insects, such as the ladybird beetles, the Monarch butterfly and the tiger moth, as well as in toxic plants such as Poppies and Ragwort (Moore *et al.*, 1990; Rothschild, 1987; Rothschild *et al.*, 1984). These findings led ecologists to suggest that MPs act as a deterring odour signal to potential predators, effectively warning of the toxicity of the host plant or insect (Rothschild *et al.*, 1984). This theory was strengthened when a study found that naive hatchling chicks displayed a neophobic (fear of something new) alerting reaction when presented with drinking water to which IBMP had been added, confirming that birds interpret IBMP as an alerting or warning signal (Guilford *et al.*, 1987), and this observation was recently confirmed in wild populations of robins (Siddall and Marples, 2011).

In grape berries MPs accumulation is greatest when the berries are unripe, therefore the role of MPs as a deterring signal seems logical as the MPs are present when it is disadvantageous to the species if the berries are eaten. As the berries ripen, and the seeds within mature, the levels of MPs decrease dramatically (Hashizume and Samuta, 1999; Ryona *et al.*, 2009) thereby making the fruit more palatable to potential seed dispersers. Considering grapes, like most fruits, emit a range of desirable attracting aroma compounds when the fruit is matured and ready to be eaten (Hardie and Obrien, 1988), it is possible that the species has also evolved to emit undesirable aroma compounds when it is not beneficial to the plant for the fruit to be eaten.

Interestingly, as well as acting as a deterrent to predators, IPMP has been found to act as a pheromonal attractant between adult ladybird beetles (Abassi *et* *al.*, 1998). As ladybird beetles are not herbivorous insects but feed on aphids and scales, it is also possible that grapevines may have evolved to produce IPMP as a way of attracting ladybirds to protect against herbivorous pests. Such tritrophic interactions have been increasingly discovered in the last two decades (for review see (Dicke, 2009)).

1.3.3 Methoxypyrazines in grapes and wine

1.3.3.1 Detection and quantification of MPs

Following the identification of MPs in vegetables, it was proposed that IBMP was responsible for the distinct vegetative/green capsicum flavour of Cabernet Sauvignon grapes and the compound was tentatively identified as a component of grapes from this variety (Bayonove *et al.*, 1975). Later IBMP and IPMP were detected in grapes of the variety Sauvignon blanc, which also produces wines with vegetative characters (Augustyn *et al.*, 1982). The increase in the sensitivity of gas chromatographic techniques, allowed both IBMP and IPMP to be quantified in Sauvignon blanc wines (Harris *et al.*, 1987), and these compounds were later confirmed to be responsible for the vegetative varietal characters of wines made from this variety (Allen *et al.*, 1995; Allen *et al.*, 1991). Subsequently, MPs have also been detected in the grapes and wines of Cabernet Sauvignon, Cabernet franc, Merlot and Carmenere, all of which are varieties that are known to produce wines which can have vegetative sensory characters that are typical of MPs (Belancic and Agosin, 2007; Kotseridis *et al.*, 1999; Lacey *et al.*, 1991). Table 1.2 shows the concentrations of MPs reported in wines of various cultivars. It is evident from these values that IBMP is the predominant MP in wines, where it is commonly found at concentrations of up to 56 $ng.L^{-1}$ in cultivars known to display vegetative characters. IPMP has been detected less often than IBMP in wines and when present tends to be at concentrations lower than IBMP. SBMP has been rarely detected in wines, and is therefore considered to be of minimal importance to wine flavour.

Table 1.2 Reported MP concentrations (ng.L^{-1}) in wines of different varieties. m indicates mean value, n indicates number of samples, nd denotes not detectable.

Variety	IBMP	IPMP	SBMP
Sauvignon blanc	0.5-38 n=22[1] 0.4– 44 n=575[2]	nd-5.0 n=22[1] nd-3.9 n=575[2]	nd -2.0 n=22[1] nd[3]
Cabernet Sauvignon	5-30 m=18 n=37[4] 3.6-56 m=19 n=12[6]	nd[5]	nd-1.9 m=0.35 n=8[6] nd[5]
Cabernet franc	6-43 m=16 n=29[4] m=14 n=6[5]		
Merlot	4-23 m=12 n=23[4] 6-12 m=8 n=12[7]	nd[5]	nd[5]
Carmenere	5-45 m=21 n=30[5]	nd-8.6 n=14[5]	

[1] (Lacey *et al.*, 1991) [2] (Alberts *et al.*, 2009), [3](Schmarr *et al.*, 2010), [4] (Roujou de Boubee *et al.*, 2000), [5] (Hashizume and Umeda, 1996), [6] (Allen *et al.*, 1994), [7] (Kotseridis *et al.*, 1999).

1.3.3.2 Sensory perception of MPs in wine

Wines that contain perceivable levels of MPs are often described as grassy, vegetative, herbaceous, earthy and green capsicum or asparagus-like. It is widely accepted that when the vegetative characters associated with MPs are at intense levels in wine they are undesirable and detrimental to the overall wine quality (Parr *et al.*, 2007). Indeed, one study has shown that in Sauvignon blanc wines the presence of MPs is negatively correlated with more desirable ripe and fruity flavours (Parr *et al.*, 2007). However, when the characters attributed to MPs are subtle and in balance with desirable fruity flavours, some consumers find their sensory perception to be enjoyable and to add important complexity to wines (Heymann and Noble, 1987). As MPs are only found in a limited number of grape varieties, particularly knowledgeable consumers regard the aromas imparted by MPs to be of great importance to the distinguishing varietal characteristics of these wines (Marais and Swart, 1999; Preston *et al.*, 2008).

The odour detection thresholds for MPs in red, white and model wines have generally been found to be the same as that in water, at 1-2 $ng.L^{-1}$ (Allen *et al.*, 1991; Maga, 1989; Pickering *et al.*, 2007). The only exception to this is IBMP, which in two separate studies was found to have a higher odour detection threshold of 15 and 16 $ng.L^{-1}$ in red wines (Maga, 1989; Roujou de Boubee *et al.*, 2000). A recent study in Cabernet Sauvignon found that when wines were spiked with either bell pepper or fruit flavours (strawberry, raspberry, blackcurrant and cherry) the intensity of these characters increased proportionately. However, when the wine was spiked with a combination of both flavours, the fruity aromas appeared to mask the vegetative aromas (Hein *et al.*, 2009). This finding may explain why in red wines, which typically display fruity characters, the odour detection threshold of IBMP has been reported at 15-16 $ng.L^{-1}$ compared with 1 $ng.L^{-1}$ in white wines and water. Another study found that within 16 Californian Cabernet Sauvignon wines, the bell pepper/capsicum character was not correlated with levels of IBMP or IPMP, suggesting that other wine components can strongly influence the perception of the characters attributed to MPs (Preston *et al.*, 2008).

Thus it appears that when the vegetative characters attributed to MPs are in balance with strong and dominating fruity flavours, their vegetative perception is subtle and adds complexity. However, if a wine is low in fruity flavours or contains an excessive amount of MPs, the vegetative flavours may become overpowering and detrimental to wine quality.

1.3.3.3 Evaluation of remedial treatments to reduce MP levels in wine

There are a number of techniques that winemakers occasionally use in an attempt to "rescue" a wine displaying undesirable flavour and aroma characteristics. The intention of these treatments is to remove the compounds responsible for the undesirable characters, known as taints. However, in doing so, these non-selective treatments often also remove desirable flavours and are therefore of limited benefit to overall wine quality. A recent study investigated the applicability of such treatments, including the addition of bentonite, activated charcoal or oak chips to wine, as well as exposure to light, for the removal of IPMP from wine (Pickering *et al.*, 2006). This study found that the addition of activated charcoal to young wine for seven days successful reduced the levels of IPMP by 34%. However, the addition of activated charcoal was not successful in reducing the asparagus and bell pepper attributes associated to IPMP and this is likely due to the simultaneous removal of other wine aromas (Lopez *et al.*, 2001). In this study no other refining treatments were successful in reducing IPMP levels, but the addition of oak chips did significantly reduce the asparagus and bell pepper characteristics associated with IPMP (Pickering *et al.*, 2006), probably due to a masking of IPMP by oak-derived flavour compounds introduced via the chips (Perez-Coello *et al.*, 2000).

Another study investigated whether different yeast strains have the ability to reduce IPMP levels during the fermentation of Cabernet Sauvignon juice (Pickering *et al.*, 2008). It was found that 3 of the yeast strains tested produced wines with similar IPMP levels while one strain actually increased the concentration of IPMP by 11% (Pickering *et al.*, 2008). Another study investigating MP levels during wine storage found there was no consistent effect on the MP levels in wines stored under different light and temperature conditions over a 12 month period (Blake *et al.*, 2010).

These same authors also investigated the effect of different wine closures on Cabernet franc and Riesling wines spiked with MPs (Blake *et al.*, 2009). Natural corks, synthetic corks and screw caps had no significant effect on MP concentrations in the spiked wines. Surprisingly, when the wine was stored in Tetra Pak cartons for 18 months, the levels of IBMP and IPMP in both wines were successfully reduced by 45% and 32% respectively (Blake *et al.*, 2009). However, it is unclear if storage in this medium also resulted in changes in desirable wine aromas, or whether the use of this enclosure would be beneficial to the marketability of a wine as Tetra Pak cartons are not a traditional wine storage container.

To date, there is no known effective treatment for reducing MP levels in wine without the potential to compromise positive attributes of the wine. It has been shown that the concentration of IBMP in a given wine is strongly correlated ($R^2 = 0.97$, $p < 0.0001$) with the concentration of IBMP in the grapes used for vinification (Kotseridis *et al.*, 1999; Roujou de Boubee *et al.*, 2002; Ryona *et al.*, 2009). Therefore, given the lack of a reliable method of removing MPs from wines, the most effective strategies for producing wines with a desirable level of vegetative characters will involve controlling the concentration of MPs present in the grapes at harvest.

1.3.4 Accumulation of methoxypyrazines in grapes

Given the importance of MPs, particularly IBMP, to the flavour and aroma of wines of certain varieties, many studies have focused on understanding the accumulation of these compounds in grapes, and how environmental and viticultural factors affect their concentration at harvest. Table 1.3 lists some concentrations of MPs reported in mature grapes of different varieties.

Table 1.3 Reported MP concentrations ($ng.kg^{-1}$) in mature grapes of different varieties. m indicates mean value, n indicates number of samples, nd denotes not detectable.

Variety	IBMP	IPMP	SBMP
Sauvignon blanc	0.6-78 m=16 n=15[1] 3[2], 3.6[3]	nd-6.8 m=1.1 n= 15[1] 0.7[3]	nd-0.6 n=15[1] nd[4]
Cabernet Sauvignon	17-54 m=37 n=12[5] 2.8-37 m=13 n=5[7] 13[4], 9.1[3] 4.2[10], 3.0[6]	4-12 m=8.5 n=12[5] 0.2[3] 0.3[6]	1.2[6]
Cabernet franc	3.6-23.7 m=10 n=13[8] 9[2]		
Merlot	14.8[3], 6.7[9]	0.4[3]	
Carmenere	15-100 m=47 n=9[10]	1.1-8.5 m=3.8 n=9[10]	
Semillon	24.2[3], 2[2]	2.1[3]	

[1] (Lacey *et al.*, 1991) [2] (Koch *et al.*, 2010), [3] (Hashizume and Samuta, 1999), [4] (Schmarr *et al.*, 2010), [5] (Battistutta *et al.*, 2000), [6] (Hashizume and Umeda, 1996), [7] (Noble *et al.*, 1995), [8] (Ryona *et al.*, 2008) [9] (Scheiner *et al.*, 2010), [10] (Belancic and Agosin, 2007).

1.3.4.1 Location of MPs within grape berries and bunches

A study investigating the location of IBMP within mature Cabernet Sauvignon berries found that grape skins contain IBMP concentrations twice that of the seeds, while minimal IBMP was found in the flesh (Roujou de Boubee *et al.*, 2002). The location of IBMP primarily within grape skins has implications during wine making as this explains why the free run juice of grapes generally has lower concentrations of MPs than pressed juice or juice macerated in the presence of skins and seeds (Roujou de Boubee *et al.*, 2002; Sala *et al.*, 2004; Sala *et al.*, 2005; Kotseridis *et al.*, 1999).

The stem (rachis) of grape bunches has also been found to contain significant levels of MPs (Hashizume and Samuta, 1997; Hashizume and Umeda, 1996). This can also be a source of MPs in wines as stems are occasionally added to fermentations to add tannin and phenolic compounds that are different from those found in the berry skins and seeds, thereby increasing the astringency and bitterness of wine (Boulton *et al.*, 1995). Interestingly, varieties which lacks MPs in the berries, such as Pinot noir and Muscat Bailey A, have been found to contain IPMP and IBMP at high levels (3.5 - 619.7 $ng.L^{-1}$) in the bunch stems (Hashizume *et al.*, 1998; Hashizume *et al.*, 2001a). The apparent organ-specific accumulation of MPs in grapevines could provide useful comparative information that will be important in understanding the biosynthesis of MPs in grape berries.

1.3.4.2 Effect of grape berry maturity on MP concentration

The concentration of MPs varies greatly according to the developmental stage of the grapes and the general trend is illustrated in Figure 1.1. The accumulation of MPs begins one to two weeks after flowering and they reach a peak in concentration approximately one week prior to véraison (véraison is a French term for colour change, used to signify the onset of grape ripening and sugar accumulation). IBMP concentrations within unripe berries have been reported at 200-247 $ng.L^{-1}$ in Cabernet franc (Ryona *et al.*, 2009; Scheiner *et al.*,

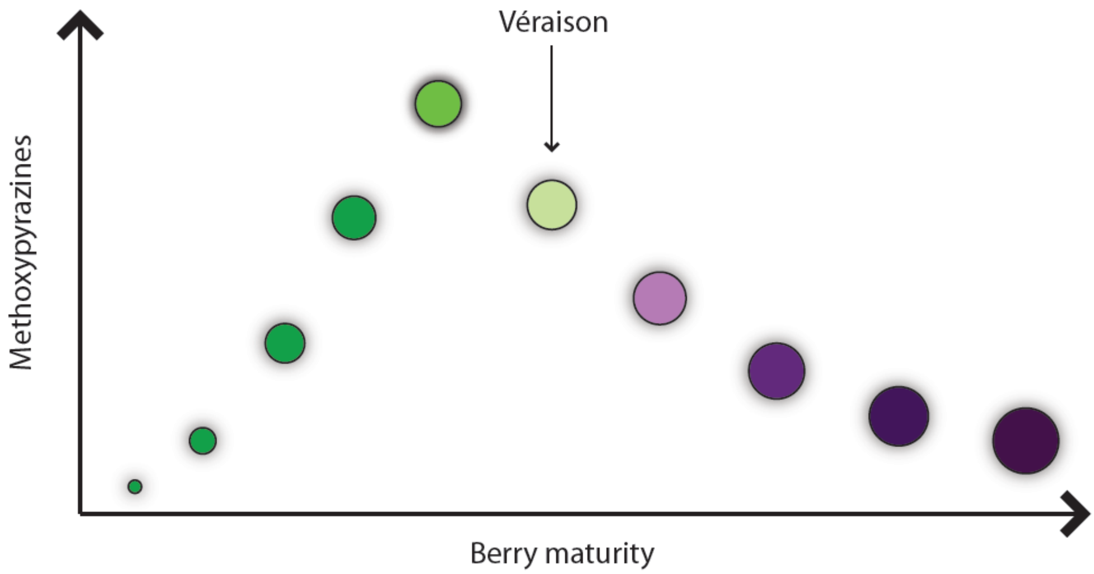


Figure 1.1 – Schematic of the typical accumulation pattern of MPs in developing grape berries. MP accumulation pattern based on the findings of Hashizume and Samuta, 1999; Koch *et al.*, 2010; Ryona *et al.*, 2009 and Scheiner *et al.*, 2010.

2010), 143-157 $ng.L^{-1}$ in Cabernet Sauvignon (Hashizume and Samuta, 1999; Koch *et al.*, 2010), and 211, 104 and 94 $ng.L^{-1}$ in Merlot, Semillon and Sauvignon blanc respectively (Koch *et al.*, 2010). Beginning at the onset of véraison, the MP content of berries declines rapidly and generally continues to decline gradually through until harvest. In mature grapes IBMP concentrations have been reported to be between 0-16% of the respective pre-véraison concentrations, and the size of this decrease is largely dependent on the length of ripening time (Hashizume and Samuta, 1999; Koch *et al.*, 2010; Ryona *et al.*, 2009; Scheiner *et al.*, 2010). In the post-véraison stages of ripening, the physical expansion of berries results in the dilution of many compounds (Conde *et al.*, 2007). However, the dilution of IBMP due to berry expansion only accounts for approximately 10% of the reduction in IBMP concentrations (Ryona *et al.*, 2009), and therefore it appears that other active processes must be involved in the reduction of MP concentrations during the ripening stages of grape berry development. Possible explanations for the decline in MP concentrations during development include further metabolism, volatilization out of the berry and possible degradation by sunlight.

1.3.4.3 Effect of sunlight exposure on the concentration of MPs in grapes

The decrease in MP concentrations during the ripening stage of berry development has largely been attributed to photo-degradation of the compounds, as it has been observed that MPs are degraded by light *in vitro* (Heymann *et al.*, 1986). The effect of sunlight exposure on IBMP levels has received much attention, as this is a factor that can be easily manipulated in the vineyard and therefore provides a potentially effective strategy for manipulating the MP content of grapes.

Indeed a number of studies have shown that increased sunlight exposure within the vineyard leads to reduced levels of IBMP (Marais *et al.*, 1999; Noble *et al.*, 1995; Ryona *et al.*, 2008; Scheiner *et al.*, 2010). The first of these studies showed that grapes from vines with low light intensity in the fruit zone, due to shading from a vigorous canopy, contained approximately 3.5-10 fold higher concentrations of IBMP than grapes from vines within the same vineyard with higher fruit zone light intensity (Noble *et al.*, 1995). However, other variables existed between the vines used for this study, including the water holding capacity, drainage and nutritional content of the soils, which were proposed to be largely responsible for the differences in the vines vigor. Another study shaded Sauvignon blanc vines by training the two adjacent vines to provide increase canopy shading of the target vine (Marais *et al.*, 1999). This study found that over two years and within three growing regions of South Africa grapes of shaded vines contained between 20 and 300% higher levels of IBMP than the non-shaded controls in the 3 weeks proceeding harvest (Marais *et al.*, 1999).

Recently more detailed studies have confirmed that sunlight exposure to berries directly results in a reduction in IBMP levels. Furthermore, these studies demonstrate that its influence is greatest during pre-véraison stages of development as opposed to post-véraison when photo-degradation of the compound was thought to occur (Ryona *et al.*, 2008; Scheiner *et al.*, 2010). Ryona *et al.* (2009) thinned Cabernet franc vines by removing shoots at flowering to produce regions of high and low light exposure to different bunches on the same vine. Exposed grape bunches were found to have 21-44% less IBMP than shaded bunches pre-véraison but no significant differences were observed at harvest. Scheiner *et al.* (2010) investigated this further by determining the effect of removing the basal leaves surrounding grape bunches at different times throughout the development of Cabernet franc berries. These authors found that removal of basal leaves from around grape bunches had the greatest effect in reducing IBMP levels when performed at 10 days after flowering, which reduced IBMP levels at harvest (125 days after flowering) by approximately 89% compared to controls. Leaf removal at 40 and 60 days after flowering also reduced IBMP levels at harvest by 68% and 46% respectively compared to control bunches. As sunlight exposure appears to have the greatest effect in reducing IBMP levels when applied early in berry development, when IBMP appears to be synthesized, it has been proposed that sunlight exposure may actually decrease IBMP synthesis rather than increase the photo-degradation of the compound (Ryona *et al.*, 2008; Scheiner *et al.*, 2010).

However, results contrary to this have also been observed. A study that used sackcloth to artificially shade Cabernet Sauvignon berries at véraison found no significant differences in IBMP concentration in the pressed juice of the grapes at harvest compared to non-shaded controls (Sala *et al.*, 2004). When the juice from these samples was macerated for one day in the presence of the skins (where the majority of grape IBMP is located, see section 1.3.4.1), the concentrations of IBMP increased approximately 4 fold and the wines produced from these grapes had a significant difference in IBMP concentrations, with the wine made from exposed berries containing 62% more IBMP than the wine made from the shaded berries (Sala *et al.*, 2004).

Another study found that when Cabernet Sauvignon berries were exposed to light, concentrations of IPMP decreased by approximately 40 % over a 120 h period. However, no significant decrease was seen in IBMP levels in the same samples (Hashizume and Samuta, 1999). When unripe grapes were used, light exposure was found to have the opposite effect and actually increased the concentrations of both IBMP and IPMP by 33% and 20% respectively. When the unripe berries were treated with $CaCl_2$, to inhibit enzymatic reactions that may be involved in the synthesis of MPs, both IBMP and IPMP levels decreased exponentially when exposed to light. These authors suggested that light exposure has two opposite effects: promoting the formation of MPs in unripe grapes; and the photo-degradation of MPs in ripening grapes (Hashizume and Samuta, 1999).

Despite these contradictory results, most of the published data suggests that light exposure in the vineyard leads to a reduction in IBMP content in berries. However, it is unclear whether this reduction is result of photo-degradation of IBMP or possibly due to other factors, such as a decrease in MP biosynthesis or possibly a result of increased thermal-degradation, as exposed berries tend to be of a higher temperature than shaded berries (Smart and Sinclair, 1976).

1.3.4.4 <u>Effect of temperature on the concentration of MPs in grapes</u>

Another environmental factor that influences MP concentrations in grapes is temperature. It has been observed that vines grown in cool climatic regions tend to produce grapes and wines with higher vegetative and herbaceous characters than grapes grown in warmer climates (Heymann and Noble, 1987). Notable examples of this are the Sauvignon blanc wines from New Zealand's Marlborough region which are often characterised by high levels of vegetative flavour (Parr *et al.*, 2007).

Indeed a number of studies have shown that the MP content of grapes is typically greater in cooler regions. One such study found that a sample of Sauvignon blanc wines from New Zealand had mean IBMP and IPMP concentrations of 25.9 and 4.4 $ng.L^{-1}$ respectively, while Sauvignon blanc wines from typically warmer Australia were significantly lower ($p < 0.001$) in IBMP and

IPMP concentrations with means of 6.8 and 1.3 $ng.L^{-1}$ respectively (Lacey *et al.*, 1991) . Similarly a strong negative correlation (R = 0.754, p < 0.005) was seen between long-term mean January temperatures and IBMP concentrations in Cabernet Sauvignon wines from both Australian and New Zealand (Allen *et al.*, 1994). The effects of temperature on IBMP accumulation has not just been observed in vineyards from different regions but also from the same vineyard over different growing seasons (Belancic and Agosin, 2007; Chapman *et al.*, 2004; Kotseridis *et al.*, 1999; Lacey *et al.*, 1991). One study measured the IBMP content of Sauvignon blanc wines made from the same vineyard over seven consecutive vintages and found that concentrations ranged from 6.4 to 38 $ng.L^{-1}$ with a relative standard deviation of 60% (Alberts *et al.*, 2009).

These studies provide strong evidence that the temperature in different grape growing regions can greatly influence the MP levels in the grapes, with lower temperatures tending to produce grapes with higher MP content. However, it is unclear if the effect of temperature on MP accumulation is through changes in the rate of biosynthesis or degradation of the compounds.

1.3.4.5 Effect of water status on the concentration of MPs in grapes

In an experiment conducted in Spain, Cabernet Sauvignon vines that were not irrigated were found to produced grapes with undetectable levels of IBMP in the juice at harvest, while 3.9 $ng.L^{-1}$ of IBMP was present in the juice of grapes from irrigated vines (Sala *et al.*, 2005). It has also been observed that growing seasons with high levels of summer rainfall are associated with increased grape IBMP accumulation (Belancic and Agosin, 2007; Roujou de Boubee *et al.*, 2000). Belancic and Agosin (2007) found that within three separate regions in Chile the IBMP concentrations of Carmenere grapes in 2004 were between 2.3 and 6.7 fold higher than in the other years of the study (2003 and 2005), which was attributed to a 3-12 fold increase in summer rainfall in 2004. The authors suggest that the increase in IBMP levels in that year of heavy summer rains was a result of increased vegetative growth leading to decreased sunlight exposure of the grapes (Belancic and Agosin, 2007). Similar observations have been observed in Cabernet Sauvignon vines of differing canopy vigour, arising from differences in the water holding capacity of the vines soil (Noble *et al.*, 1995). These studies display the difficulty in determining the ultimate cause of berry compositional changes as viticultural experiments are often confounded by the interaction between variables.

1.3.4.6 <u>Effect of grape yield on the concentration of MPs in grapes</u>

Grape yield has also shown to have an impact on the IBMP levels of grapes (Chapman *et al.*, 2004). In this study, Cabernet Sauvignon vines were pruned to produce six treatments differing in the number of buds per vine, and thus bunches per vine. Across two seasons significant (p < 0.001) negative correlations of R^2 = 0.7385 and 0.7007 were seen between buds per vine and the IBMP concentration in the resulting wines. In this study the vines with the lowest yield (12 buds per vine) produced wines with approximately double the IBMP concentration than the highest yielding vines (48 buds per vine). The authors noted that because of the pruning method employed the low yielding vines had fewer shoots and leaves than those with higher yields and consequently the bunches experienced more light exposure than those on the higher yielding vines (Chapman *et al.*, 2004). Therefore, despite the increase in light exposure, which previous experiments suggests leads to a reduction in MP levels (section 1.3.4.3), wines made from low yielding vines still accumulated significantly higher levels of IBMP than wines made from high yielding vines. This suggests that fruit yield has a direct influence on IBMP levels independent of sunlight exposure. This may also explain the observations made in cooler climates (section 1.3.4.4) where both canopy growth and yield may be reduced.

1.3.4.7 Site of MP biosynthesis within grapevines

As the MP content of berries appears to be, in part, influenced by yield and vine vigor, it had been noted previously that the biosynthesis of MPs could occur in the leaves of grapevines and the compounds translocated into the berries via the xylem (Noble *et al.*, 1995; Ryona *et al.*, 2008). However, this theory was recently disproven by Koch *et al.* (2010). These authors employed a reciprocal grafting experiment using Cabernet Sauvignon vines, which do accumulate MP in the berries, and Muscat blanc vines, which do not accumulate MP in the berries. It was found that when Muscat blanc grape bunches were grafted onto vines of Cabernet Sauvignon the Muscat blanc grapes still did not accumulate IBMP, while the Cabernet Sauvignon grape bunches that were grafted onto Muscat blanc vines retained the ability to accumulate IBMP. These authors concluded that the accumulation of IBMP is dependent on the genotype of the berry, where IBMP is likely to be synthesised, and therefore not translocated to the berry from elsewhere in the vine (Koch *et al.*, 2010).

1.3.5 Ladybird beetle as a source of MP in wine

Although grapes provide the source of MPs in the vast majority of wines that it is encountered in, there is one other known source of MPs in wine. The invasive ladybird beetle *Harmonia axyridis* has been known to infest vineyards where they feed on aphids and coccids living on the grapevine. The ladybird, which secretes IPMP as a pheromonal attractant (See section 1.3.2) can be incorporated into the winemaking process along with the harvested grapes, thereby tainting the wine with large quantities of IPMP (Pickering *et al.*, 2006). Native to Asia the ladybird beetle is most notorious as a wine taint in North America where it was introduced in 1916 as a biological control for aphids and coccids (Brown *et a*l., 2008). *Harmonia axyridis* is also rapidly spreading in Europe but it is not currently established in Australia (Brown *et a*l., 2008). Although ladybirds can drastically influence a wine by adding large amounts of undesirable IPMP, the number of occurrences is minimal in context of global wine production and therefore will not be expounded on further.

1.3.6 Pathway of methoxypyrazine biosynthesis

Despite the interest in identifying factors that influence MP accumulation in grapes, little work has been done in determining the pathway of MP biosynthesis. This is true in not just grapes but any plant species. However, a biosynthetic pathway for MPs has been proposed (Fig. 1.2). This hypothetical pathway begins with the reaction of an amino acid and an unknown 1,2-dicarbonyl compound leading to the formation of a 3-alkyl-2-hydroxypyrazine (HP) intermediate, which is then enzymatically methylated to form a MP (Leete *et al.*, 1992; Murray *et al.*, 1970). In the bacterial strain *Pseudomonas perolens*, which accumulates high levels of IPMP, feeding experiments have shown that the addition of ^{13}C labelled valine results in the production of ^{13}C labelled IPMP, confirming that amino acids are a precursor to MPs in bacteria (Gallois *et al.*, 1988). It is thought that the amino acids valine, leucine and isoleucine are the precursors of IPMP, IBMP and SBMP respectively, because of similarities in the alkyl side chains of the compounds, as shown in Figure 1.3 (Murray and Whitfield, 1975).

Despite evidence that amino acids are a precursor to MPs it remains unclear by what mechanisms the given amino acid is converted to the HP intermediate. It has been proposed that the respective amino acid gains a second nitrogen through an unknown amidation reaction before undergoing a condensation reaction with an unknown 1,2-dicarbonyl compound to produce a HP (Murray *et al.*, 1970). Glyoxal is the 1,2-dicarbonyl compound commonly used in the chemical synthesis of MPs, however no reports could be found indicating its presences in grape berries. Two structurally similar compounds, acetaldehyde and glycoaldehyde, which are by products of pyruvic acid metabolism (Sweetman *et al.*, 2009) and tartaric acid biosynthesis (DeBolt *et al.*, 2006) respectively, could potentially be precursors in the formation of HPs in grape berries.

The final step in the proposed pathway, involving the methylation of HP to form MP, is thought to be catalysed by an *O*-methyltransferase enzyme. Indeed, a study in grapevines found that Cabernet Sauvignon berries contain enzymatic activity that is capable of methylating HPs to form MPs *in vitro* (Hashizume *et al.*, 2001a).

1.3.7 Grapes contain a methyltransferase enzyme that methylates HPs

Extensive literature searches found that the only known previous investigations into the biosynthesis of MPs in a plant species were performed in grapevines. In the first of these studies, it was demonstrated that crude protein extracts from unripe grape berries and grapevine shoots contained enzyme activity capable of performing the final methylation step in the putative pathway of MP biosynthesis

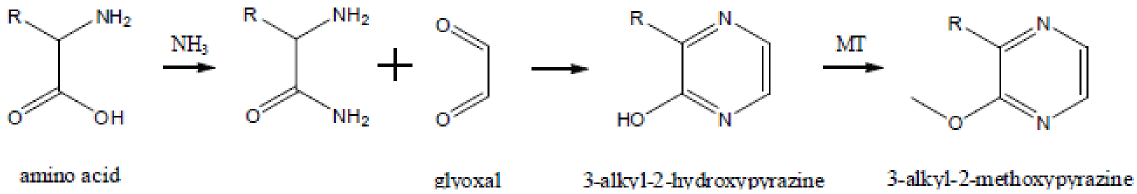


Figure 1.2 - The proposed biosynthetic pathway of MPs (Leete *et al.*, 1992; Murray *et al.*, 1970).

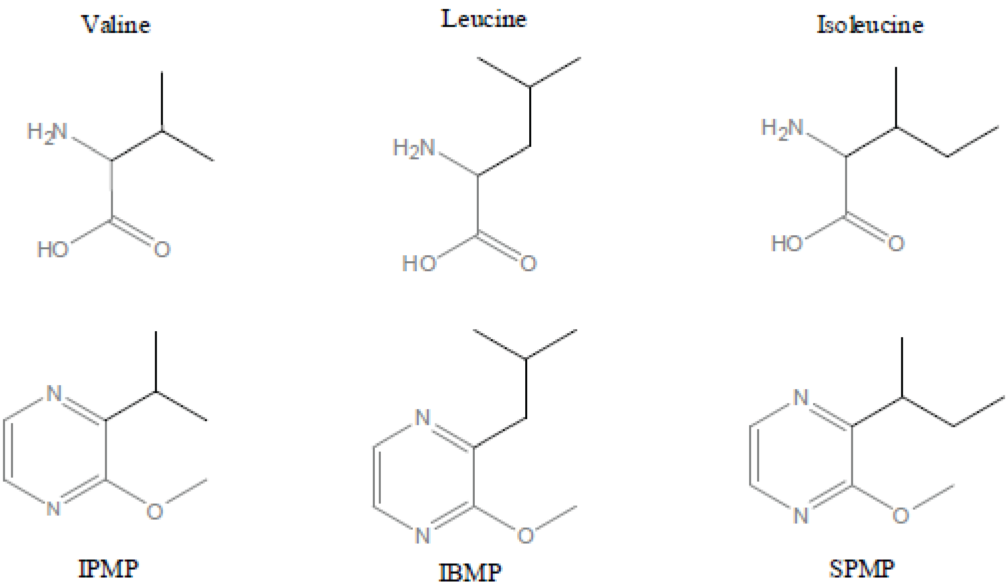


Figure 1.3 - Similarities between the alkyl side chains of MPs and their proposed amino acid precursors.

(Hashizume *et al.*, 2001a). Furthermore, these authors demonstrated that there is a strong association between the amount of HP methylating activity and the subsequent levels of MPs in the unripe berries of eight different cultivars (Fig. 1.4 A&B). Interestingly, the varieties Muscat Bailey A and Pinot noir, which were found to contain only trace levels of MPs, also contained only minimal levels of HP methylating activity (Hashizume *et al.*, 2001a). This finding suggests that some grape varieties do not accumulate MPs in berries because they are unable to perform this final step in MP biosynthesis in berry tissues.

In this same study, 3-isobutyl-2-methoxypyrazine (IBHP) and 3-isopropyl-2-hydroxypyrazine (IPHP) substrate concentrations were quantified in the unripe grape samples and were found to be between 2 and 20-fold higher than the corresponding MP concentrations in all cultivars (Fig. 1.4 C). This suggests that the enzymatic methylation of HP to MP is a rate-limiting step in the production of MPs, making it a good target for the manipulation of the MP content of grape berries.

In a subsequent study, the same authors purified an *O*-methyltransferase enzyme with HP-methylating activity from Cabernet Sauvignon shoots and

sequenced 22 amino acids of its N-terminus (Hashizume *et al.*, 2001b). *In vitro* functional assays of the purified *O*-methyltransferase showed that this enzyme is multifunctional and has the ability to methylate each of the HP precursors of IPMP, IBMP and SBMP. In addition to methylating HP substrates, this *O*-methyltransferase showed activity against a number of other hydroxyl containing molecules and was found to have a ten-fold greater binding affinity against caffeic acid than against HPs (K_ms of 0.3 and 0.032 mM respectively). This implies that HPs are not the preferred substrates of this OMT and that the

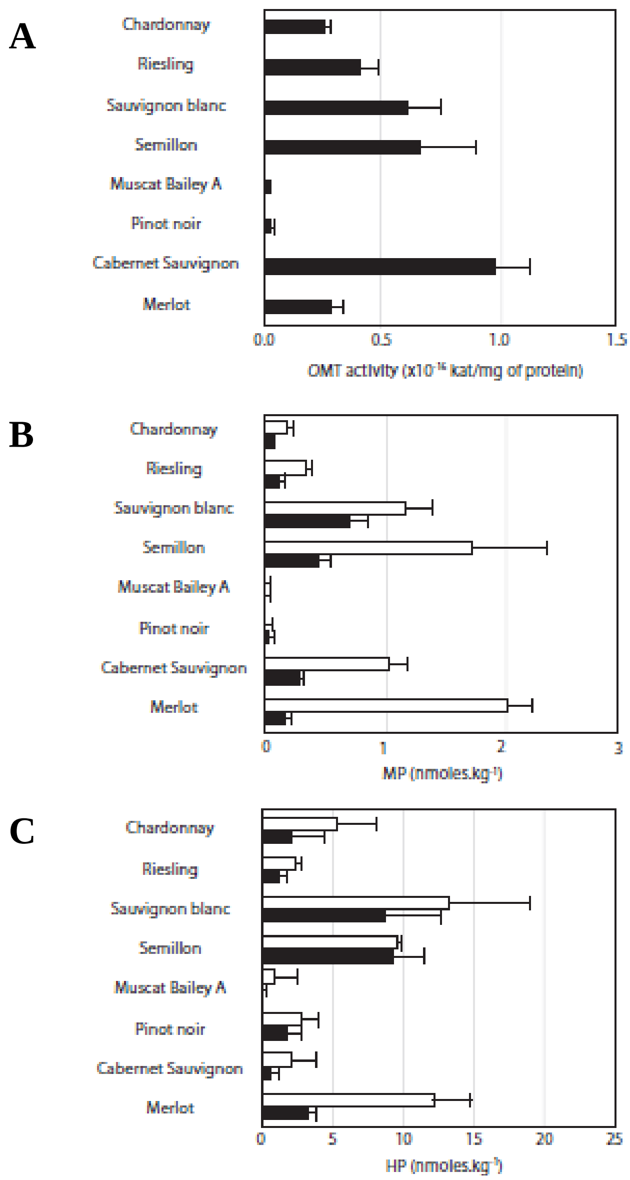


Figure 1.4 – **A)** HP methylating activity in crude protein extract from immature grapes of eight different varieties. **B**) Concentrations of IBMP (white bars) and IPMP (black bars) measured in the respective varieties, and C) concentrations of the precursors IBHP (white bars) and IPHP (black bars). Figures redrawn from Hashizume *et al.* (2001a).

biosynthesis of MPs in grapes may be a by-product of another metabolic pathway. This could have implications with regards to the flux of MP biosynthesis during grape berry development and in response to external stimuli if the nature of the primary pathway this OMT functions in is known.

1.4 Project aims

MPs are grape derived aroma compounds with highly odorous vegetative characteristics which, when present in wine at high levels, can have a detrimental effect on wine quality. MPs are present in only a few grape cultivars including, Sauvignon blanc, Cabernet Sauvignon, Merlot and Semillon, yet these four varieties account for approximately 29% of the total wine grapes currently grown in Australia (Source: Australian Wine and Brandy Corporation).

The final concentration of MPs in grape berries at harvest appears to be determined by the balance between the amount that is synthesised pre-véraison and the amount that is degraded/metabolised post-véraison. While there is growing knowledge of how viticultural and environmental factors can alter the MP content of berries, the metabolic processes responsible for these changes in MP concentrations during berry development and in response to various stimuli remains unclear.

The primary aim of this research project was to identify and characterise the *O*-methyltransferase gene or genes responsible for the final step in the pathway of MP biosynthesis in grapes. In addition, this project aimed to improve our understanding of MP accumulation in grape berries, and to investigate how the final rate limiting methylation step is affected by environmental factors and viticultural management practices known to influence MP concentrations in berries. This knowledge will lead to a greater understanding of the key determinants of MP biosynthesis and accumulation in grapes, ultimately allowing better management decisions to be made in the vineyard to produce grapes and wines of a desired MP content to meet consumer demands, as well as applications for the breeding of new grape varieties.