Organoleptic Impact of 2-Methoxy-3-isobutylpyrazine on Red Bordeaux and Loire Wines. Effect of Environmental Conditions on Concentrations in Grapes during Ripening

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The 2-methoxy-3-isobutylpyrazine content in grapes and red wines was assayed by stable isotope dilution gas chromatography−mass spectrometry, following vapor extraction and purification on a cation resin microcolumn. The threshold beyond which the green bell pepper character is marked in wines has been determined. From a comparison of the 2-methoxy-3-isobutylpyrazine concentrations of 50 red Bordeaux and Loire wines from different vintages and grape varieties (Cabernet Sauvignon, Cabernet franc, and Merlot) with the intensity of the green bell pepper character as perceived on tasting, the threshold value was estimated to be 15 ng/L. Statistical analysis of the 2-methoxy-3-isobutylpyrazine concentrations of 89 red Bordeaux wines showed that Cabernet wines were more commonly affected by this vegetative character. Changes in the 2-methoxy-3-isobutylpyrazine concentration as the grapes ripen are affected by the environmental and cultural conditions (soil, climate, training system, etc.). A very good correlation was shown between the breakdown of malic acid and 2-methoxy-3-isobutylpyrazine as the grapes ripened, irrespective of grape variety, type of soil, or weather conditions.

Keywords: *2-Methoxy-3-isobutylpyrazine; assay; organoleptic impact; Cabernet Sauvignon; ripening; environmental and cultural conditions; malic acid*

INTRODUCTION

Initially identified in green bell pepper (*Capsicum annuum*) (Buttery et al., 1969), 2-methoxy-3-(2-methylpropyl)pyrazine (isobutylmethoxypyrazine) is a highly odorous compound. Its perception threshold in water has been estimated at 2 ng/L (Buttery et al., 1969). Isobutylmethoxypyrazine (IBMP), first detected in Cabernet Sauvignon grapes by Bayonove et al. (1975), is considered to be responsible for the vegetative, green bell pepper aromas found in certain wines made from a number of *Vitis vinifera* grape varieties: Cabernet Sauvignon, Cabernet franc, Merlot, and Sauvignon Blanc (Augustyn et al., 1982; Heymann et al., 1986; Harris et al., 1987; Calo et al., 1991; Allen et al., 1991, 1993, 1994; Lacey et al., 1991; Kotseridis et al., 1998a,b).

Although this odor was generally considered by wine tasters as detrimental to the quality of red wine aroma, very little data were available in the literature linking a wine's IBMP content and the intensity of its green bell pepper character, as perceived on tasting.

It has, however, been established in several of the world's wine-growing regions that the IBMP concentration in grapes drops during ripening and that this phenomenon depends on climate, the vine's vegetative growth, and vineyard management techniques affecting the sun exposure of the grapes (Lacey et al., 1991; Hashizume et al., 1996; Allen et al., 1996). It therefore seemed interesting to investigate whether this compound could act as a marker for grape ripeness. This paper aims to address the following issues: (a) more closely defining the relationship between the perceived intensity of IBMP in wines and its actual content; and (b) investigating the influence of vineyard environmental conditions (soil type and training system as well as climate) on the IBMP content of grapes during ripening.

MATERIALS AND METHODS

Wines and Juices. The must and wine used to calibrate the assay were from Sémillon and Ugni Blanc grapes. Further analysis did not present IBMP in the wine from Ugni Blanc and presented only a slight amount in the must from Sémillon. The calibration was therefore corrected by subtracting the blank ratio (area formed by ion *m*/*z* 167 of the IBMP contained naturally in this must/that of internal standard).

The red Bordeaux wines analyzed were made from Cabernet Sauvignon, Cabernet franc, and Merlot grapes from several different vintages. The Loire wines (1991 and 1992 vintages) were Cabernet franc from an estate in the Saumur Champigny appellation.

Analysis of the IBMP content during ripening of the grapes was carried out using ∼1 kg of grapes taken from each plot in a random, yet representative, manner. The grapes were then frozen at −20 °C. They were then defrosted, dried with absorbent paper, and crushed in a kitchen mixer (Philips, type HR2875) for 1 min. The crushed grapes were left to macerate for 2 min and then blended for a further 30 s. The must was centrifuged (3000 rpm, 15 min), and the clarified juice was treated with 50 mg/L SO_2 and stored in opaque bottles (Nalgène bottles, code 20520) at −20 °C prior to analysis.

Sensory Analysis. The detection threshold of IBMP in a model solution (ethanol at 12% volume, 5 g/L tartaric acid,

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pH 3.5) was determined by triangular tests (Boidron et al., 1988) with a panel of 25 people from the Faculty of Enology (Université Victor Segalen Bordeaux II). Subjects were instructed to smell the three glasses of the triangular test and to indicate the odd sample.

Statistics. PCSM (Deltasoft) software was used for the statistical analyses.

Experimental Plots. In 1996, variations in the IBMP concentrations in Cabernet Sauvignon grapes during ripening were measured on three plots in the Bordeaux area, located in the Pessac-Léognan (PL), Saint Emilion (SE) (both on gravel soil, vines trained in vertical shoot positioning, guyot pruned, and density of 7500 plants/ha), and Entre-Deux-Mers (E2M) (on sandy-silt soil, vines trained in vertical shoot positioning, guyot pruned, and density of 3500 plants/ha) appellations. In 1997, the same experiments were carried out on Cabernet Sauvignon and Merlot grapes from Pessac-Léognan (on gravel soil, vines trained in vertical shoot positioning, guyot pruned, and density of 7500 plants/ha).

Assessment of Grape Ripeness. Grape ripeness in the plots studied was monitored from véraison to harvest, using standard must analyses for reducing sugars and malic acid. Meteorological data (temperature, hours of sunshine, and precipitation) were provided by the INRA weather station at La Grande Ferrade (Villenave d'Ornon, France).

Chemicals and Glassware. The 2-methoxy-3-isobutylpyrazine was guaranteed 99.9% pure by the supplier (Pyrazine Specialities, Inc., Atlanta, GA). The deuteriated internal standard, 2-($^2H_3$)methoxy-3-isobutylpyrazine, was synthesized using the protocol described by Harris et al. (1987). The dichloromethane used throughout these experiments was ultrapure (purity > 99.95%, Atrasol, SDS, France; code 02935E). All of the glassware used in the isolation of the methoxypyrazine was washed with chromic acid (minimum 24 h at 20 °C), then rinsed with water and ultrapure water (Milli-Q, Millipore), and, finally, dried in a heat chamber (100 °C). The cation-exchange resin (AG50W-X4, Bio-Rad) was used as received.

Methoxypyrazine Extraction. The wine or must sample (250 mL) plus 20 ng of the internal standard [2-($^2H_3$)methoxy-3-isobutylpyrazine] and 10 mL of NaOH at 10% was left to balance for a minimum of 1 h. IBMP was then extracted using the process devised by Allen et al. (1994), modified as follows:

Distillation of the must or wine at atmospheric pressure was replaced by steam extraction, with a system frequently used in enology laboratories for measuring volatile acidity and alcoholic strength in wine (Œnoextracteur Chenard, Lamothe Abiet Pinosa, patent no. 84 17403). It consists of extracting volatile compounds of the wine introduced in the bubble chamber by the steam during 4.5 min. The steam wass then passed through a stainless steel and Rachy ring rectifying column connected to a stainless steel cooler with lateral manifold, providing condensation of the distillate. Approximately 180 mL of distillate was collected. This rapid method extracts between 85 and 100% of the IBMP in the sample, as compared to ~6% by distillation (results not shown).

Instead of agitating the distillate in the presence of cation-exchange resin, the extract was percolated through a column, in the following way. The ion-exchange resin (~1 g) was in a Pasteur pipet with a glass wool plug in the narrow section. The resin was rinsed (5 mL of ultrapure water), and then the steam extract was percolated through it by means of a peristaltic pump (10 mL/min). When percolation was completed, the circuit was rinsed with 5 mL of ultrapure water. The column was disconnected from the circuit, and IBMP was eluted with 1 mL of 10% NaOH solution. The eluate was collected in a conical flask. Finally, the pipet was rinsed with 200 μL of ultrapure water and then 500 μL of dichloromethane collected with the eluate. The mixture was agitated in a vortex system, and then the organic phase that separated out was transferred to a 1 mL vial. The aqueous phase was extracted again using 500 μL of dichloromethane. The organic phases were put together, dried on anhydrous sodium sulfate (Merck, Darmstadt, Germany), evaporated to <10 μL under a nitrogen stream at constant temperature and pressure, and, finally, analyzed by GC-MS.

Table 1. Repeatability of Methoxypyrazine Analysis in Cabernet Sauvignon Wine (Bordeaux, 1995)

sample	3-isobutyl-2-methoxypyrazine (ng/L)
1	15.1
2	14.8
3	15.7
4	14.8
5	16.5
av (n = 5)	15.4
SD	0.66
CV (%) (5%)	4.3

GC-MS Analysis. The system used was a Varian Star 3400 Cx coupled with a Varian Saturn 2000. The capillary column was a BP 20 (50 m × 0.22 mm i.d.; 0.25 μm film thickness; SGE). The vector gas was helium N55 (>99.999%; Air Liquide), with a flow rate of 53 mL/min. Injection was made in splitless mode. The temperature gradient from 60 °C (initial isotherm for 1 min) to 110 °C was 5 °C/min and then 3 °C/min up to 230 °C, finally isothermal for 6 min. The detector (ionization energy = 70 eV) operated at 250 °C and was only switched on 12 min after injection started. Each injection lasted 57 min. Detection was carried out in CI with ammonia, selecting an ion range of m/z between 165 and 172, thus including both isobutylmethoxypyrazine (m/z 167) and the internal standard (m/z 170).

Calibration. The system was calibrated by adding increasing concentrations of IBMP (2−40 ng/L for must and 0−76 ng/L for wine). The internal standard concentration was 80 ng/L. Each of the samples prepared in this way was extracted according to the method previously described.

Repeatability. The variation coefficient was determined by five analyses (extraction and measurement) of the IBMP content of five samples of the same wine made from Cabernet Sauvignon grapes in 1995.

RESULTS

Standard Curves and Repeatability. The standard curves for the assay were obtained using samples of must or wine to which increasing concentrations of IBMP had been added. The area of the IBMP peak (m/z 167) compared to that of the peak of the internal standard (m/z 170) was in linear correlation with the IBMP concentration of the sample. The regression equations for must and wine are, respectively: [IBMP] (ng/L) = $80.141(A/A_{is}) - 3.492$ ($r^2 = 0.988$) and [IBMP] (ng/L) = $78.609(A/A_{is}) - 1.2636$ ($r^2 = 0.984$) (A, area of IBMP peak; A_{is}, area of internal standard peak).

The repeatability of the IBMP analysis on a series of five measurements is shown in Table 1, with the associated statistical values. For a wine containing an average of 15 ng/L IBMP, the assay method was repeatable to within 4.3%.

Intensity of the Green Bell Pepper Character Perceived on Tasting a Wine and Its IBMP Content. The organoleptic impact of IBMP in red wines had not been clearly identified in previous works. The detection threshold of IBMP in a model solution was found to be 6 ng/L.

The recognition threshold in wine is only an indicative value, due to the great variations in composition from one wine to another. This value is useful, however, as it indicates the concentration above which the odor produced by the compound under investigation is identified (Boidron et al., 1988). The following procedure was used to determine a threshold for IBMP representative of a specific type of wine beyond which the green bell

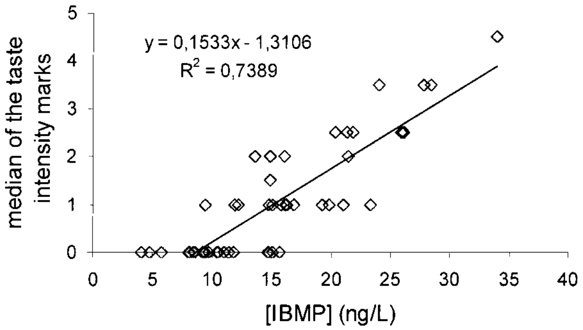

Figure 1. Correlation between the median of the taste intensity marks obtained for each of the 50 red Bordeaux and Loire wines and the IBMP concentration determined by GC-MS analysis.

Table 2. Distribution of IBMP Concentration (in Nanograms per Liter) in Bordeaux Wines Made from Cabernet Sauvignon (CS), Cabernet Franc (Cf), and Merlot (M) Grapes in Different Vintages

grape variety	no. of samples	$[IBMP]_{min}$	$[IBMP]_{max}$	$[IBMP]_{av}$[a]	SD
CS	37	5	30	18a	5.3
M	23	4	23	12b	4.0
Cf	29	6	34	16a	6.2

[a] Statistically significant differences by Newman−Keuls test (5%) are indicated by different letters.

pepper character is marked. Fifty red Bordeaux and Loire wines were ranked on a scale of 0−5 according to the intensity of their green bell pepper character by a 10-person tasting jury at the Bordeaux Faculty of Enology. The median of the taste intensity marks obtained for each of the wines was calculated with Excel 97 (Microsoft Corp.). The relationship between the median of the flavor intensity marks obtained for each of the wines and its IBMP content was linear ($r^2 = 0.74$) (Figure 1).

Wines with no green bell pepper character (median = 0) contained an average of 10 ng/L IBMP; those considered to have weak green bell pepper (median = 1), 15 ng/L; and above that value the perception of this character was medium to strong. The threshold of IBMP beyond which the green bell pepper character is marked in the type of wine studied can therefore be estimated at 15 ng/L.

Distribution of the IBMP Concentration in Bordeaux Wines According to Grape Variety. The IBMP concentration of 89 Bordeaux wines from different vintages made from Cabernet Sauvignon (CS), Cabernet franc (Cf), or Merlot (M) grapes was determined (Table 2).

Most of the Merlot wines had very little methoxypyrazine, whereas both Cabernet Sauvignon and Cabernet franc had much higher concentrations. Among the wines analyzed, 70% of the Cabernet Sauvignon, 52% of the Cabernet franc, and 13% of the Merlot had IBMP contents above the previously defined threshold.

A chi-square test was used to determine the statistical relationship between the "grape variety" and "IBMP concentration" variables. For this purpose, the range of IBMP concentrations in the 89 wines studied was divided into five classes established by the software (0/6.8, 6.8/13.6, 13.6/20.3, 20.3/27.1, and 27.1/33.9 ng/L). The results revealed a statistically significant relationship between the two variables at a threshold of 5%. In other words, the various IBMP concentrations in different wines seems to be related to the grape variety. A variance analysis was then carried out on the averages. Once the variances for the three grape varieties had been checked using a Log-ANOVA test, we studied the equality or nonequality of the averages (see Table 2) by comparing the variability within each sample with the variability between samples (Fisher test). The averages with overall significant differences were also analyzed (significance threshold = 5%). To obtain more accurate results, the averages were compared among themselves using a Newman−Keuls test with a threshold of α = 5%. There were statistically significant differences between the following groups: Merlot/Cabernet Sauvignon and Merlot/Cabernet franc. Only the Cabernet Sauvignon/Cabernet franc group was not statistically distinguishable. The green bell pepper aroma produced by IBMP may, therefore, be considered a varietal aroma of Cabernet wines.

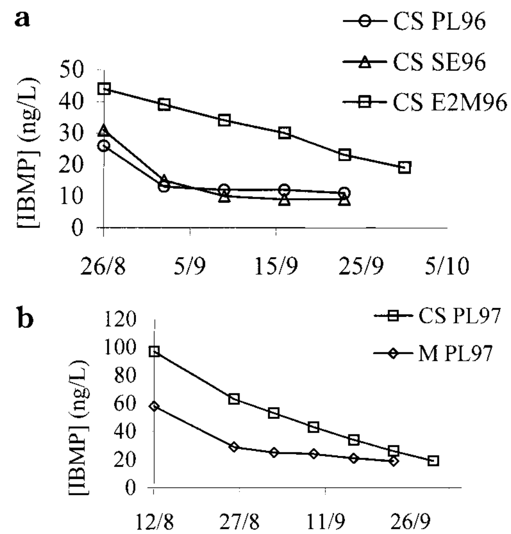

Figure 2. Variations in the IBMP concentration of grapes during ripening in three different environmental conditions in 1996 [(a) Pessac-Léognan (PL), Saint Emilion (SE), and Entre-Deux-Mers (E2M)] and in one Pessac-Léognan vineyard in 1997 (b). CS, Cabernet Sauvignon; M, Merlot.

Variations in the IBMP Contents of Cabernet Sauvignon and Merlot Grapes in the Bordeaux Area under Different Environmental and Cultural Conditions. This comparison was carried out in 1996 (Figure 2a) on three plots of Cabernet Sauvignon grapes grown on two types of environmental and cultural conditions (sandy-silt soil, 3500 plants/ha; and gravel soil, 7500 plants/ha). The grapes had different IBMP concentrations at the end of the véraison in the various plots. The value was higher (45 ng/L) in plots with sandy-silt soil. Variations in the IBMP contents of the grapes during ripening also differed from plot to plot. On gravel soils, the IBMP content dropped very rapidly and then stabilized at a low level ($\approx$10 ng/L), below the previously defined threshold, approximately two weeks before the harvest. On the plot with sandy-silt soil, the breakdown kinetics were much more gradual. The grapes contained twice as much IBMP when they were picked than those grown on gravel, with values above the threshold of 15 ng/L.

Figure 2b compares the variations in IBMP contents of Merlot and Cabernet Sauvignon grapes, grown on a plot with gravel soil in the Pessac-Léognan appellation, under the weather conditions of the 1997 vintage. During the ripening process, the IBMP content of the

Table 3. Comparison of Weather Conditions (Average Monthly Temperature, Monthly Sunshine Hours, and Monthly Precipitation) between June and October in 1996 and 1997[a]

	av monthly temp (°C)		monthly sunshine hours (h)		monthly precipitation (mm)	
	1996	1997	1996	1997	1996	1997
June	15.5	17.6	190.2	196.3	86	144
July	20.7	18.5	250.5	172.7	64	150.5
Aug	21.3	21	226.3	210.8	71	46.5
Sept	20	23.7	175.5	199.1	141.5	106.5
Oct	16.5	19.8	177.8	198.3	103.5	38.5
total	94	100.6	1020.3	977.2	466	486

[a] Data provided by the INRA weather station, La Grande Ferrade-Villenave d'Ornon.

Table 4. Determination Coefficient (r^2) between the Breakdown of Malic Acid and IBMP during Ripening in Cabernet Sauvignon (CS) and Merlot (M) Grapes Grown on Different Soils in 1996 and 1997

1996	r^2	1997	r^2
CS Saint Emilion	0.90	CS Pessac-Léognan	0.91
CS Entre-Deux-Mers	0.96	M Pessac-Léognan	0.99

Merlot grapes was always lower than that of the Cabernet Sauvignon grapes. However, the values measured when the grapes were picked were in the vicinity of the threshold of 15 ng/L or higher. Under comparable soil and cultural conditions, concentrations were twice as high as in 1996, a sunnier, less humid year (Table 3).

Correlation between the Breakdown of Malic Acid and IBMP in Grapes during Ripening. In both 1996 and 1997, the malic acid content was determined for each of the grape samples used in IBMP analysis, from véraison to harvest. The variations in both of these parameters during the period under study were then correlated (Table 4). We show that the breakdown of malic acid and IBMP occurred simultaneously, irrespective of soil type, grape variety, or vintage. Like malic acid, IBMP may therefore be considered to be a marker for grape unripeness.

DISCUSSION AND CONCLUSION

The modifications to the IBMP assay in grapes and wines (steam extraction and percolation on a cation resin microcolumn) made sample preparation faster and more convenient. The assay as described makes it possible to analyze relatively large series with good repeatability.

The literature has very little data linking the methoxypyrazine content of wines and the intensity of the green bell pepper character perceived on tasting. By adding increasing amounts of IBMP to a bulk wine, made of unspecified grape varieties, Maga (1989) found that the recognition threshold for this component was 16 ng/L. Using a duo–trio difference test, Allen et al. (1991) assessed the perception threshold of IBMP in an Australian white blended wine at 2 ng/L. Using a sample of 50 red wines (Bordeaux and Loire) from different vintages, made of Cabernet Sauvignon, Cabernet franc, and Merlot grapes, we have shown that these types of wines generally had a marked green bell pepper character with an IBMP content of 15 ng/L.

The independence of the variables was studied for 89 red Bordeaux wines, combined with variance analysis, and Cabernet wines were shown to be more frequently affected by these vegetative odors. IBMP concentration was >15 ng/L in only a minority of the Merlot wines.

The influence of climatic parameters (temperature, hours of sunshine) on the IBMP content of grapes during ripening is well-known (Lacey et al., 1991; Allen et al., 1996). The comparisons in the literature describe wine-growing regions that are relatively far apart, with quite different climates. We show that, under comparable climatic conditions in the Bordeaux vineyards, the methoxypyrazine contents of the grapes at véraison and variations during ripening are strongly influenced by the environmental and cultural conditions (type of soil, pruning and training system, density of plantation, etc.) in each specific plot. These results are in agreement with the frequent appearance of a marked vegetative character in Cabernet Sauvignon wines from Bordeaux vineyards grown on sandy-silt soils with a low planting density. This behavior of Cabernet Sauvignon grapes in these conditions is clearly identifiable, even in years when climatic conditions are favorable for the grapes to ripen properly.

In comparable plots (gravel soil, high planting density) within the Bordeaux vineyards, variations in the Atlantic climate from one vintage to another also affect the IBMP content of the grapes during ripening. In a more humid vintage, such as 1997, the grapes had a higher IBMP content throughout the ripening period, although there was more sunshine and higher September and October temperatures than in 1996 (these parameters were identical in August). In 1997, June and July were marked by frequent rainfalls (+144.5 mm compared to 1996). A direct result was the reconstitution of the soil water reserves, which favored an important growth of the vine until the harvest. That is why in 1997, although September and October were warmer, sunnier, and less humid than 1996, grapes ripened in worse conditions.

Finally, this study showed a very good correlation between the breakdown of malic acid and IBMP in grapes during ripening. It has been clearly established that a high malic acid content in grapes is a marker of poor ripening (Peynaud, 1946). The IBMP content may be considered to have the same significance.

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