# Determination of 3-alkyl-2-methoxypyrazines in grapes, musts and wines: a review

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## Abstract

3-alkyl-2-methoxypyrazines are aromatic compounds present among the flavors of a wide range of foods, such as bell peppers, asparagus, peas, and potatoes. Some of these compounds have also been found in Cabernet sauvignon, Sauvignon blanc, Cabernet franc and Merlot noir grapes and wines. Although their contents in these samples are at ng/L level, they can influence wine aroma because of their low sensory thresholds. Identification and quantification of 3-alkyl-2-methoxypyrazines is challenging because it demands very sensitive analytical techniques. Since 3-isobutyl-2-methoxypyrazine was found in grapes of Cabernet sauvignon for the first time, different methods have been reported in the literature. The aim of this paper is to give an overview on them and the results obtained. The application of isolation and concentration techniques such as liquid-liquid-, solid-phase- and solid-phase microextraction is discussed.

## Keywords

3-alkyl-2-methoxypyrazines, analysis, aroma, determination, flavor, grape, must, nitrogen compounds, odorant, *Vitis vinifera*, wine

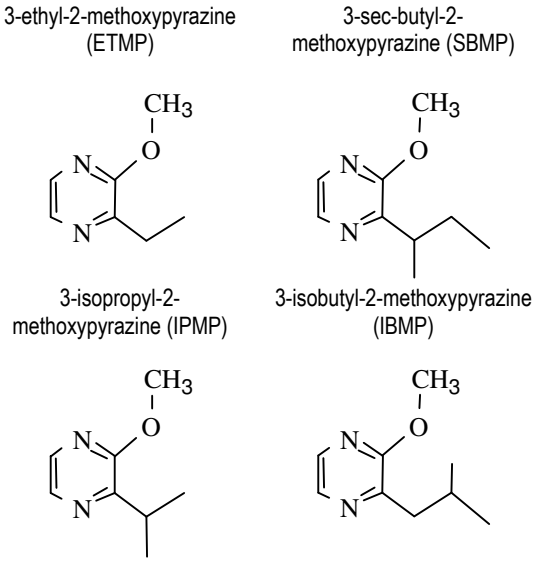
## Introduction

Pyrazines (Figure 1) are often found in nature. Most of them have strong smells and contribute to the flavor of fresh and cooked foods *(1)*. Some pyrazines are used in food aromas and in perfumes *(2)*. 3-alkyl-2-methoxypyrazines (MPs) tend to have green and vegetative aromas and they are often found in fresh vegetables, like potatoes, peas, green peppers and asparagus *(2)*. The most important MPs in wines are 3-isobutyl-2-methoxypyrazine (IBMP), 3-*sec*-butyl-2-methoxypyrazine (SBMP), and 3-isopropyl-2-methoxypyrazine (IPMP) (Figure 1). These compounds, especially IBMP, have been found in the flavor of several wine varieties of great economical importance: Sauvignon blanc, Cabernet sauvignon, Cabernet franc and Merlot noir. These varieties are used in the production of high quality wines all over the world. Consequently, the study of their varietal aromas is of great interest to the field of enology.

Although sensory evaluation is crucial for analyzing wine flavor and quality, instrumental measurements can play an important role in quantifying them and therefore looking at differences that may be too subtle to be noticed by means of sensory analysis. However, such subtle differences might be useful to detect improvements in viticulture or winemaking techniques. Thus, the availability of a reliable methodology to quantify these aromas in musts and wines is of great potential interest to grape growers and winemakers. This review summarizes the different techniques used to determine the 3-alkyl-2-methoxypyrazines in grapes, musts and wines, discussing the difficulties faced and the results obtained so far.

MPs, and particularly IBMP, SBMP and IPMP have extraordinarily low sensory thresholds, at the ng/L level in wines *(3-5)* and they occur at such low levels in grapes and wines.

**Figure 1:** Main 3-alkyl-2-methoxypyrazines found in grapes, musts and wines.




The complexity of the wine matrix together with the extremely low contents at which MPs occur are a challenge to the analyst. This is because it involves isolating, detecting, identifying and quantifying very scarce aroma compounds within a mass of other substances, which may be present at concentrations several orders of magnitude higher. Therefore, this labor has to be done at the limits of equipment capacity, and it requires very rigorous research methodologies. Indeed, analytical techniques at ultra-trace levels need a high

concentration capacity and a very good clean-up of the sample.

A major problem with analysis of MPs in grapes and wines has been the lack of sensitivity of the available analytical techniques. This challenge is so difficult that, although the first data tentatively indicating the presence of IBMP in Cabernet sauvignon grapes was published in 1975 *(6)* and several authors were afterwards encouraged to work on this research *(7-9)*, the first report of a reliable method was published more than 10 years later *(10)*. This procedure was subsequently improved by different authors *(11-14)*. More convenient methods have been published *(15-17)*, some of them taking the advantages of the solid-phase microextraction (SPME) technique for the extraction of volatile compounds *(18-20)*. The application of isolation and concentration techniques such as liquid-liquid-, solid-phase- and solid-phase micro- extraction are discussed below, together with the chromatographic conditions and the analytical parameters of the methods.

## Isolation and concentration methods

Due to the difficulties of ultra-trace analysis, the reported methods are often based on combinations of clean-up, isolation and concentration techniques. Most of them are based on liquid-liquid extraction (LLE) and solid-phase extraction (SPE).

Their main characteristics are summarized in Tables 1 and 2 and discussed below. Finally, the SPME technique is discussed at the end of this section.

***LLE.*** This is the simplest and most generally used technique for analyzing aroma compounds of foodstuffs. The main problem of its application to the determination of MPs in grapes, musts and wines is that it requires big concentration factors and other compounds present in the sample appear as interferences. Thus, it is recommended to use complementary separation techniques to clean the extract, together with very sensitive and specific detector systems.

The solvents used for the determination of MPs are Freon 11, dichloromethane, diethyl ether and the mixture diethyl ether / hexane (Table 1). These solvents are the most generally used in the analysis of wine aromas since the extraction of ethanol and water is minimal and their low boiling points allow a further concentration step.

*Freon 11.* . The methods based on LLE with Freon 11 are not specific for MPs but general methods for the analysis of aroma components from grapes, musts and wines, which made possible finding MPs among other aroma compounds. A technique based on the LLE of grapes with Freon 11 allowed the detection of ETMP, IBMP and, with less reliability, IPMP in grapes of Sauvignon blanc, for the first time *(8, 21)*. A complex method based on the LLE with Freon 11, extraction and concentration at low-temperature and high vacuum, was the first that reported the determination of IBMP in wines *(22)*. The results obtained indicated that there was an unusually high level (500 ng/L) of IBMP in a Bordeaux wine, actually the highest level ever reported, and there is a general feeling in the related literature that it was an error of determination. It was reported that another method based on the LLE of wine with Freon 11, concentration by rectification column and analysis by GC-MS-GCO allowed the identification of IPMP and IBMP in red wines *(23)*.

*Dicloromethane.* This solvent was used to extract a very large amount (170 L) of wine with 12 successive extractions. It was the first attempt to isolate and identify IBMP in a Cabernet sauvignon wine with a strong 'herbaceous' aroma *(7)*. In spite of its low sensory threshold and characteristic 'bell pepper' smell, IBMP was not detected, perhaps because the compound was lost in the complex process of extraction and concentration.

*Diethyl-ether.* An easy and fast procedure for the determination of IBMP in red wines has been developed. The method is based on a simple extraction with diethyl ether followed by concentration by $N_2$ stream *(16)*. The performance of this procedure is based on the use of the isotopic dilution technique, using a deuterated analogue of IBMP as internal standard..

**Table 1.** Main parameters of the reviewed liquid-liquid extraction techniques for determining MPs in grapes, musts and wines.

| Solvent | | Sample | | Concentration | Final Volume (µL) | CF* | Ref. |
|---|---|---|---|---|---|---|---|
| Type | Volume (mL) | Type | Volume | | | | |
| freon 11 | 50 | grapes | 2 x 250 g | rectification column | 30-50 | 5000 | *(8)* |
| freon 11 | 250 | wine | 95 mL | low temperature, high vacuum distillation | 25-75 | 1267-3800 | *(22)* |
| freon 11 | 2 x 250 | wine | 1100 mL | rectification column | 1.1-110 | 10-1000 | *(23)* |
| diethyl ether | 3 x 25 | wine | 200 mL | vacuum distillation, $N_2$ stream | 200 | 2000 | *(16)* |
| diethyl ether / hexane | 4 + 2 + 2 | wine | 50 mL | $N_2$ stream | (4 x) 200 | 62.5 | *(15)* |
| diethyl ether / hexane | 3 x 5 | grapes, wines | wine: 250 mL, berries: 1000 g | $N_2$ stream | 100 | 1000 | *(17)* |

* CF = initial volume of the sample / final volume of the extract.

*Diethyl-ether / hexane.* Two similar methods for the analysis of IBMP in red wines based on the LLE with diethyl-ether/hexane (1:1, v/v) followed by concentration under $N_2$ stream have been reported. The main difference between them is the internal standard: one of them uses the MEMP *(15)* and the other method uses a deuterated analogue of IBMP *(17)*. The advantages of these methods are the simplicity and rapidity. In these methods dichloromethane, diethyl-ether, dichloromethane/pentane (1:2, v/v) and diethyl-ether/hexane (1:1, v/v) were studied. Although the solvents diethyl-ether and dichloromethane had the best recoveries, the mixture diethyl-ether / hexane was chosen because it presented the lowest affinity for the compounds that created interferences and its level of emulsion was the smallest.

***SPE.*** Polar, non polar and cation exchange extraction techniques have been used for the isolation and concentration by SPE of MPs present in grapes, musts and wines. The phases used with the different types of extraction are, respectively: silica (Si), octadecyl (C18) and strong cation exchange with benzenesulfonic acid (SCX). The main parameters of the reported SPE techniques are summarized on Table 2. SPE demands a preliminary clean-up step to avoid the saturation of the resins. Distillation can be used to remove the less volatile interfering compounds present in the wine medium. Thus, the combination of distillation and SPE has produced some of the most successful methods for the analysis of MPs in grapes, musts and wines.

*Si.* This phase has been used to identify IBMP in Cabernet sauvignon grapes by means of a complex method *(6, 24)*. The method consists of vacuum distillation of crushed berries, LLE of the distillate with pentane, SPE with Si, concentration and analysis.

*C18.* The quantitative analysis of IBMP and IPMP in wines has been approached by means of C18 SPE *(9)*. The method consists on the steam distillation of wine at pH 5, collecting the distillate in an acidic solution and extracting with a C18 cartridge. This method could only be applied to spiked white wines due to the relatively high detection limits of the procedure (at the µg/L level) and the presence of interferences from the volatile phenols, which co-eluted with IBMP *(25)*.

*SCX.* This phase has been used in different SPE methods taking the advantage of both the volatility and the basicity of MPs *(10)*. These methods are based on the use of a strongly acidic resin which is effective in trapping them from the distillate. All of them have been applied successfully to the determination of MPs in wines. The original method is rather complex and includes the distillation of wine at pH 5-6 and SPE with a SCX resin. After elution with water at pH 10 and extraction with dichloromethane, the final extract is concentrated and analyzed. The high concentration factor allows the determination of MPs below their sensory threshold, using a relatively small (200-300 mL) sample of wine. Despite the low recoveries achieved (10-15%), the method is accurate due to the use of deuterated IBMP as internal standard. This approach allowed the identification and quantification of IPMP, IBMP and SBMP in a Sauvignon blanc wine. The major drawbacks of the procedure are the complicated sample preparation and also the fact that the internal standard used was not commercially available and had to be synthesized.

**Table 2.** Main parameters of the reviewed SPE techniques for determining MPs in grapes, musts and wines.

| Phase | Sample | | Pre-treatment | Elution | Concentration | Final Vol. (µL) | CF* | Ref. |
|---|---|---|---|---|---|---|---|---|
| | Type | Volume | | | | | | |
| Si | grapes | 35 Kg | vacuum distillation and LLE (pentane) | ethyl ether/pentane (1:1 v/v) | | | | *(6)* |
| C-18 | wine | 500 mL | adjust to pH 5 and steam distillation with acid trap | methanol | HPLC (reversed phase) | 5000 | 100 | *(9)* |
| SCX | wine | 200 mL<br>300 mL | distillation atmospheric pressure<br>dynamic head-space | water, pH 10 | LLE (dichloromethane) and spontaneous evaporation | 10 | 20000 | *(10)* |
| SCX | wine | 240 mL | distillation atmospheric pressure | water, pH 10 | LLE (dichloromethane) and spontaneous evaporation | 20 | 12000 | *(31)* |
| SCX | grapes, wines | grapes: 1 kg; wines: 300 mL | distillation atmospheric pressure | water, pH 10.5 | LLE (dichloromethane) and evaporation under $N_2$ stream | 5 | 60000 | *(12)* |
| SCX | grapes, musts, wines | grapes: 1 kg; musts and wines: 250 mL | steam distillation | solution 10% NaOH | LLE (dichloromethane) and evaporation under $N_2$ stream | 10 | 25000 | *(14)* |

* CF = initial volume of the sample / final volume of the extract.

Subsequently, several authors have based their works on this method and some of them have reported their improvements. The extraction with a higher amount of SCX resin *(12, 14)* provided higher percentages of recovery. The use of a non-deuterium-labeled alkyl-pyrazine as internal standard *(12)* resulted in a more convenient method. Finally, the steam distillation provided higher sensitivity *(14)*.

***HS-SPME.*** This technique has been applied to food analysis *(26-27)*, including the determination of wine aroma compounds *(28-30)*. Its concentration capacity, together with the selectivity of the nitrogen-phosphorous detector has proven to allow the reliable quantification of MPs in musts and wines at the levels they naturally occur in these samples *(18-19)*.

In different studies about the capacity the different SPME fibers have to extract the MPs, the following ones have been tried: polyacrylate (PA), polydimethylsiloxane (PDMS), polydimethylsiloxane-divinylbanzene (PDMS-DVB), carboxen-polydimethylsiloxane (CAR-PDMS), carbowax-divinylbenzene (CW-DVB), and divinylbenzene-carboxen-polydimethylsiloxane (DVB/CAR/PDMS) *(18, 20)*. In aqueous solutions, the PDMS-DVB fibers had the best performance *(18)*, whereas in model solutions containing 12% ethanol, the best results were obtained with DVB/CAR/PDMS and CW/DVB fibers *(20)*. Such results prove the influence of ethanol on the extraction efficiency: detection limits are of about 0.1-1 ng/L in a water-based medium, whereas in the presence of ethanol, they are of around 100 ng/L. It is therefore clear that, when applied to wines, care must be taken with ethanol since this compound strongly competes with the MPs for the fiber, resulting in very low recoveries when it is present in the sample *(20)*.

Consequently, when applied to wines, the HS-SPME technique demands a preliminary clean-up step in which ethanol is removed by low temperature distillation of the acidified sample in order to prevent its interference. Wine samples of Cabernet sauvignon and Merlot noir have been successfully determined by means of a procedure based on this principle *(19)*. This method takes the advantage of the fact that MPs are protonated at pH levels below 2.0 and their volatility decreases. Thus, the sample is distillated at low temperature to remove the ethanol, so that the protonated MPs remain in the residue. Finally, the ethanol-free solution is neutralized and the SPME is performed. The SPME technique has also been used to determine the contents of MPs in musts *(18)*. In this case, the distillation step is not required. The main advantages of the methods to determine MPs in musts and wines by means of SPME are simplicity, convenience and rapidity.

## Chromatographic conditions

Ultra-trace analysis demands very sensitive methods and consequently the authors dealing with such determination have adjusted their chromatographic conditions to achieve the maximum possible sensitivity and selectivity. Detection systems, columns and injection conditions used in the main GC methods for determining MPs in grapes, musts and wines are summarized on Table 3 and discussed below.

*Injection.* Splitless mode is generally chosen in the analysis of MPs in grapes, musts and wines. To increase the sensitivity of the chromatographic system, the following techniques have been used: programmed temperature vaporization injector (PTV) *(23)*, retention gab of deactivated column (to accommodate 3-6 µL injections) together with the cool on-column injection *(10)*, and the solvent effect *(10)*.

*Columns.* As Table 3 shows, poly(ethylene glycol) columns are the most generally used in the analysis of MPs, followed by poly(5%-diphenyl-95%dimethylsiloxane) columns. The comparison of the retention times obtained from columns with different stationary phases can allow the identification of compounds by GC. This technique has been used in a method for determining MPs in musts and wines by GC-NPD: two different columns, CP-WAX 57 CB and SPB-35, have been used to confirm the identification of the MPs *(18-19)*. The same principle was used when IBMP was identified in Cabernet sauvignon grapes for the first time *(6)*.

## Instrumental detectors

Due to its lack of selectivity, FID has not been very successful for the analysis of MPs. This was already noticed in the first report about the occurrence of MPs in grapes: the peak of IBMP obtained with a FID from a concentrate of 35 kg of must of Cabernet sauvignon was too small to be quantified *(6)*. The main use of the FID in the analysis of MPs in grapes, musts and wines is as a complement to GCO *(6-8, 23)*.

Although NPD is very sensitive, its detection limits are not low enough for the analysis of MPs by direct injection of the sample. Consequently, it requires a procedure for concentrating the target compounds before analyzing them by GC. This procedure should provide high recoveries and a high concentration factor. Despite the selectivity of this detector, a good clean-up is also necessary since NPD can fail to detect the MPs if the amounts of other compounds are too high *(8)*. The GC-NPD together with the HS-SPME technique has been successfully applied to the analysis of MPs in musts and wines *(18-20)*. NPD has also been used as a complement of GCO instead of FID since it allows the identification of the peaks *(8)*.

The relatively high detection limits of MSD demand a method that provides a great concentration factor. The first identification of IBMP in Cabernet sauvignon was not successful due to the lack of sensitivity of MSD *(6)*. A similar problem happened to the first identification of IBMP in grapes of Sauvignon blanc: although the concentration factor was high, MP contents in the injected extracts were close to the detection limits of the MSD *(8)*.

The selected ion monitoring (SIM) mode is a must in the application of MSD to the analysis of MPs in grapes, musts and wines, since the total ion chromatogram (TIC) mode results in a lack of sensitivity *(7, 15-17, 22)*. The first reliable determination of MPs was provided by the combination of the MSD in SIM with both electron ionisation (EI) and chemical ionisation (CI) *(10)*.

**Table 3**. Chromatographic conditions used in the reviewed GC methods for determining MPs in grapes, musts and wines.

| Injector | Column | Detector | Clean-up | Analytes | Ref. |
|---|---|---|---|---|---|
| splitless | CW 20 M (23m, 0.25mm i.d.) | MSD | LLE Freon 11 | flavor compounds | *(8)* |
| splitless | DB-WAX (50m, 0.25mm i.d.) | FID, NPD MSD (EI, CI) | LLE Freon 11 | flavor compounds | *(22)* |
| PTV | DB-WAX (60m, 0.32mm i.d., 0.5µm) | MSD (EI), GCO | LLE Freon 11 | flavor compounds | *(23)* |
| | CW 20 M, glass capillary, 44m | MSD, FID | LLE dichloromethane | flavor compounds | *(7)* |
| | CW 20 M (127m, 0.75mm i.d.). | FID | LLE dichloromethane | flavor compounds | *(7)* |
| | CW 20 M, glass (305cm, 4mm i.d.) | FID, GCO | LLE dichloromethane | flavor compounds | *(7)* |
| splitless | CW 20 M (50m, 0.25mm i.d., 0.2µm) | MSD (EI) | LLE ether/hexane | IBMP, C-6-alcohols, β-damascenone, α- and β-ionone, free terpenols | *(15)* |
| splitless | CW 20 M (50m, 0.25mm i.d., 0.2µm) | MSD (EI) | LLE ether or ether/hexane | IBMP, α- and β-ionone, β-damascenone | *(16-17)* |
| cool on-col. retention gab. | BP20 (25m, 0.33mm i.d., 1.0µm) | MSD (EI, CI) | SPE SCX | IBMP | *(10)* |
| cool on-col. retention gab. | DB-Wax (30m, 0.32 mm i.d., 0.5 µm) | MSD (EI, CI) | SPE SCX | IPMP, IBMP | *(31)* |
| splitless | DB-WAX (60m, 0.32 mm i.d., 0.25µm) | MSD (EI) | SPE SCX | IPMP, SBMP, IBMP | *(12)* |
| splitless | CP-WAX 57 CB (50 m, 0.25 mm i.d., 0.2 µm ) | NPD | HS-SPME | MP, MEMP, ETMP, IPMP, SBMP, IBMP | *(18-19)* |
| splitless | BP 20 (50 m x 0.22 mm i.d.; 0.25 µm) | MSD (CI) | SPE SCX | IBMP | *(14)* |
| cool on-col. retention gab. | DB-1 (60 m, 0.32 mm i.d., 1.0 µm) | MSD (EI, CI) | SPE SCX | IPMP, IBMP | *(31)* |
| cool on-col. retention gab. | DB-1701 (30 m, 0.32 mm i.d., 1.0 µm) | MSD (EI, CI) | SPE SCX | IPMP, IBMP | *(31)* |
| T: 20°C, 6 s, 200°C/min to 190°C | BP-5 (50 m, 0.32 mm i.d., 1.0 µm) | MSD (EI), GCO | LLE Freon 11 | flavor compounds | *(23)* |
| cool on-col. retention gab. | BP5 (25m, 0.33 mm i.d., 0.5 µm) | MSD (EI, CI) | SPE SCX | IBMP | *(10)* |
| cool on-col. retention gab. | BP5 (50m, 0.32mm i.d.,1.0µm) | MSD (EI, CI) | SPE SCX | IPMP, IBMP | *(31)* |
| splitless | HP-5MS (30m, 0.25mm i.d., 0.25µm) | NPD | HS-SPME | ETMP, IPMP, SBMP, IBMP | *(20)* |
| splitless | SPB-35 (30m, 0.25mm i.d., 0.25µm) | NPD | HS-SPME | MP, MEMP, ETMP, IPMP, SBMP, IBMP | *(18-19)* |
| splitless | Reoplex 400 (150m, 0.46mm i.d.) | MSD, FID | LLE Freon 11 | flavor compounds | *(8)* |
| split 30:1 | Reoplex 400 (65m, 0.25mm i.d.) | FID, NPD | LLE Freon 11 | flavor compounds | *(8)* |
| splitless | SE-54 (30m, 0.25mm i.d.) | FID, NPD MSD (EI, CI) | LLE Freon 11 | flavor compounds | *(22)* |
| | SE-30 glass capillary, 60 m | MSD | LLE dichloromethane | flavor compounds | *(7)* |

Some modifications of this method used only the CI in SIM *(14)*. EI could be applied instead of CI since the higher recoveries achieved with several improvements of the method allowed doing so *(12)*.

**Olfactometry**

The technique of gas chromatography-olfactometry (GCO) consists on the replacement of the instrumental detector by a human nose. The outlet of the column is split and one part is lead to an instrumental detector to allow recording the peaks and the other part is conditioned in terms of temperature and humidity to allow the sniff analysis of a trained expert.

The extremely low levels at which the analytical work has to be performed are the major challenge to the analyst when dealing with the determination of MPs in grapes, musts and wines. However, the low sensory thresholds of these compounds have been cleverly used by means of GCO. Indeed, the fact that the human nose is more sensitive than the available detection systems has been used by several authors in order to detect the MPs in the complex extracts of the samples analyzed.

GCO was crucial to the first detection and identification of IBMP in musts of Cabernet Sauvignon. The authors tentatively identified IBMP as the responsible for the green 'pepper-like' smell characteristic of the variety. Although the identification by MSD was not fully reached, they could confirm that the retention time and the smell of the compound were the same as the ones of the standard IBMP in five different chromatographic columns *(6)*. Similarly, the comparison of the smell retention times of some extracts with the standards of MPs confirmed the identification of ETMP, IPMP and IBMP in grapes of Sauvignon blanc, although these compounds could not be quantified neither with NPD nor with MSD *(8)*.

The same way as the detection of the characteristic smells of the analytes by GCO may contribute to confirming their identification, the absence of these characteristic smells at their corresponding retention times can be considered evidence that these compounds are not present in the analyzed extracts. In spite of its low sensory threshold and characteristic 'bell pepper' smell, IBMP was not detected by GCO in a concentrated extract obtained from 170 L of Cabernet sauvignon wine, suggesting that the MPs were not present in the concentrated extract *(7)*.

GCO has also been used to compare the performance of two alternative techniques for the extraction of aroma compounds: LLE and an alternative headspace technique. The later proved to be less efficient because the aroma intensities detected by GCO were hardly detectable or significantly lower *(8)*.

Finally, the analysis by GCO allows extracting information regarding the relative strength of the smell of each compound. This is performed by means of the aroma extract dilution analysis technique, which consists on analyzing by GCO the concentrated extract at several successive dilutions. This information is of great interest because it can link the chemical analysis with the sensory analysis. This technique has been applied to the analysis of the aroma of some young red wines made with Merlot noir, Cabernet sauvignon and Grenache grape varieties and it was reported that IPMP and IBMP were among the most important odorants of the wines analyzed *(23)*.

## Analytical parameters

The determination of MPs in grapes, musts and wines has historically been a challenge to the analysts and a lot of work is still needed in order to achieve fully optimized and validated methods. Internal standards, recoveries, limits of detection, linearity and reproducibility reported on the methods reviewed here are summarized on Table 4 and discussed below.

*Internal Standards*. The correct choice and use of the internal standard is always crucial, but in the case of ultra-trace analysis it is even more important. The use of acetophenone, chemically different from MPs, might have been responsible for a possible bias on the determination of IBMP in a Bordeaux wine *(22)*. The different types of internal standards used for the determination of MPs in grapes, musts and wines can be classified as follows: alkyl-pyrazines, 3-alkyl-2-alcoxypyrazines and isotopically labeled analogues of MPs.

It has been reported that contents of 500 ng/Kg of TMP were present in Japanese wines *(33)*. However, no further reports on the presence of this compound in wines have been published during the last three decades and TMP has been used as internal standard for quantifying MPs in wines *(9)*. Nevertheless, when considering the use of this compound as internal standard, it should be taken into account that it has been reported that it is not stable under steam distillation conditions *(9)*. 2-methyl-3-*n*-propyl-pyrazine has been used as an alternative to the isotopically labeled internal standard, although not being chemically identical to the target compounds *(12)*.

Due to the similarity of their chemical structure to the target compounds, 3-methyl-2-methoxypyrazine, 3-ethyl-2-ethoxypyrazine and 3-isopropyl-2-ethoxypyrazine have been used as internal standards *(15, 18-20)*.

The main advantages of the stable isotope dilution method are its simplicity and accuracy. With this technique, the internal standard and the target compound are chemically identical because the analyte itself is the internal standard. Interference from natural isotopic compounds can be prevented by the use of bi- and tri-deuterated analogues. MSD allows to distinguish the added standard from the natural component. Under the appropriated chromatographic conditions, the isotopically labeled IBMP elutes 2-3 seconds before the IBMP *(10)*. The use of 3-isobutyl-2-[$^{2}H_{3}$]-methoxypyrazine as internal standard was key to the first reliable method for determining MPs in grape juices and wines *(10)* and several modifications of this method have used the same internal standard *(14, 31-32)*. Later on, one of the improvements of this method included the simultaneous use of two trideuterated analogues of MPs: 3-isobutyl- and 3-isoproppyl-2-[$^{2}H_{3}$]-methoxypyrazine *(11)*. Subsequently, an easy method for the synthesis of a bideuterated analogue of IBMP, the 3-[1,1-$^{2}H_{2}$]-isobutyl-2-methoxypyrazine, was reported *(16)*. Since the synthesis of this compound is easier, this method simplifies the use of a deuterium labeled internal standard and it has been successfully applied to the analysis of wines *(17)*.

*Recovery*. Data on recovery tends not to be reported the literature, with the exception of four methods. The authors of the first reliable method for determining MPs in grape juices and wines mentioned that only around 5-10% was recovered *(10)*. Later on, a modification of this method provided a better 86-103% of recovery *(12)*. A method based on the LLE by means of ether-hexane proved to have an excellent performance, with a 90% recovery *(17)*. Finally, the percentages of recovery of a methodology based on the HS-SPME were high for IPMP, SBMP and IBMP (78-109%) in musts and wines *(18-19)*. Care must be taken, though, in the interpretation of the recoveries obtained with the SPME technique because they can not be directly compared with the results obtained from the LLE, since the SPME technique does not allow the determination of the concentration of the extract.

*Limits of detection.* The concentration factor provided by the method, the sensitivity and selectivity of the detector and the recoveries play an important role to allow reaching the order of magnitude at which MPs occur in grape and wine samples: around 10 ng/mL. If detection limits are too high, the determination is not possible *(9, 20, 22)*. By means of a very sensitive detector, limits of detection of around 2 ng/L have been obtained with relatively low concentration factors *(15-17)*. Finally, limits of detection below 1 ng/L require concentration factors higher than 10,000 *(10-12, 18-19)*.

**Table 4**. Analytical parameters of the reviewed GC methods for determining MPs in grapes, musts and wines.

| Internal standard Compound | ng/L | Recovery (%) | DL* (ng/L) | Ref. |
|---|---|---|---|---|
| Acetophenone | 20[a] | | 1000 | *(22)* |
| 3-[1,1-$^2H_2$]-isobutyl 2-methoxypyrazine | 2500[b] | | 2 | *(16)* |
| 3-methyl-2-methoxypyrazine | 2264[b] | | 2 | *(15)* |
| 3-[1,1-$^2H_2$]-isobutyl 2-methoxypyrazine | 20 | 90 | 2 | *(17)* |
| Tetramethylpyrazine | | 53 (IBMP); 14 (IPMP) | 1200 | *(9)* |
| 3-isobutyl-2-($^2H_3$)-methoxypyrazine | 33.2 | 5-10 | 0.1 (MEMP) | *(10)* |
| 3-isobutyl- and 3-isopropyl- 2-($^2H_3$)-methoxypyrazine | IBMP: 103.2 IPMP: 75.1 | | 0.15 | *(11)* |
| 3-isobutyl- 2-($^2H_3$)- methoxypyrazine | 80 | | | *(14)* |
| 2-methyl-3-*n*-propyl-pyrazine | 100 | 86 – 103 | <0.5 (IBMP); <1.0 (IPMP, SBMP) | *(12)* |
| 3-isopropyl-2-ethoxypyrazine | 10 | 94-109 (IPMP, SBMP, IBMP); 43-58 (MP, ETMP, MEMP) | 0.1 (IPMP, SBMP, IBMP); 0.5 (ETMP); 1 (MEMP, MP) | *(18)* |
| 3-isopropyl-2-ethoxypyrazine | 10 | 78-105 (ETMP, IPMP, SBMP, IBMP); 31-36 (MP,MEMP) | 0.3 (IPMP, SBMP, IBMP); 1 (ETMP), 4 (MEMP, MP) | *(19)* |
| 3-ethyl- and 3-isopropyl-2-ethoxypyrazine | 20000 | | 100 | *(20)* |

* DL: Detection limit. (a) added to the concentrate (b) calculated from the data provided in the report.

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