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3-Methyl-2-butene-1-thiol: Identification, analysis, occurrence and sensory role of an uncommon thiol in wine

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ABSTRACT

A highly uncommon odorant, 3-methyl-2-butene-1-thiol was detected by using Gas Chromatography-Olfactometry (GC-O) and unequivocally identified for the first time in wine. A purge and trap sampling technique which provides highly representative extracts for olfactometric analysis was used for the extraction of the volatile fraction of a Spanish red wine made from Prieto Picudo grapes. The identification of the odorant was achieved by multidimensional gas chromatography analysis of the same purge and trap extract. Mass spectrum and retention indices in both polar and non-polar columns allowed knowing unequivocally the identity. To obtain quantitative data a method was validated for the analysis of the compound at ng L^{-1} level with acceptable precision. This powerful odorant presented an odor threshold in wine of 0.5–1 ng L^{-1} and it has been detected in several Prieto Picudo wines at concentrations slightly above the odor threshold.

1. Introduction

Wine is one of the foods with the most complex aroma from a chemical point of view. In a wine, between 20 and 40 different substances have a role in the aroma perception and these odorants appear together with other volatiles (several hundreds) in a too low concentration to be perceived. This makes the analysis and understanding of wine aroma an extremely difficult task.

To face this problem, a step by step protocol must be followed. First of all, it is absolutely essential to know all the odorants that are likely to exert a significant role in wine aroma, screening them from the wide array of volatile non-odor active compounds. The best tool to achieve that is Gas Chromatography-Olfactometry [1,2]. However results are strongly dependent on the extraction technique used and it is essential a technique that provides representative extracts. Different extraction techniques have been developed and optimized [3–5] to recover a representative proportion of active odorants, and although a perfect technique does not exist, a high representativeness has been demonstrated for some of the designs described earlier [6,7]. Another essential aspect is the identification of odorants detected in olfactometry. Most of the compounds that contribute to wine aroma are already known however during the last years some new odorants present at very low concentrations but playing a potentially sensory role have been detected and identified [8–10]. For the correct identification of these molecules it has been shown that the development of multidimensional gas chromatography (MDGC) coupled with mass spectrometry and olfactometry is a beneficial approach. MDGC provides a high selectivity and allows hearth-cut for both identification as well as micro-preparative purposes [11].

The second necessary step to understanding wine aroma is to know the concentration of important odorants in wine. A large number of methodologies have been developed depending on the nature and concentration level of the target analytes. Some analyses of ultra-trace odorants require several concentration and separation steps, eventually incorporation of derivatization reactions and highly sensitive and selective detection methods. This means that very specific methods are needed for the analysis of just a few minor but important trace-level compounds.

Finally, the sensory role of potentially important odorants has to be checked by various sensory tests, both in synthetic and in real wine. The odor thresholds of an odorant in a given matrix are a useful parameter to know if an analyte will be sensory active at a given concentration. Furthermore, addition, suppression, reconstitution or other similar test must be done in order to know the effect of a compound or group of compounds in a complex mixture [12] and to study perceptual interaction between odorants [13].

Another way to face this last aspect is to try to relate the sensory description of a wine with its quantitative composition by using statistic tools. In the literature, there have been various methods described, being Partial Least Square Regression (PLSR),

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Principal Component Analysis (PCA) and other modeling procedures the most commonly applied [14,15].

In this work a comprehensive study has been carried out to identify, quantify and study the sensory role of a so far unknown odorant detected in a red wine made from Prieto Picudo grapes.

2. Materials and methods

2.1. Reagents and standards

The chemical standards were supplied by Sigma (St. Louis, USA), Aldrich (Gillingham, UK), Fluka (Buchs, Switzerland), Lancaster (Strasbourg, France), PolyScience (Niles, USA), Chemservice (West Chester, USA), and Firmenich (Geneva, Switzerland). 3-methyl-2-butene-1-thiol (MBT) (1% soluction in triacetin) was from Frutarom (Seaton Carew, UK). Hexane (UniSolv), dichloromethane (SupraSolv), methanol (SupraSolv) and ethanol (gradient grade for liquid chromatography; LiChrosolv) were from Merck (Darmstadt, Germany). For instrumental analysis diethyl ether was from Fluka (Buchs, Switzerland), anhydrous sodium sulfate was for analysis ACS-ISO quality from Panreac (Barcelona, Spain), ethylenediaminetetraacetic acid disodium salt 2-hydrate (EDTA), L-cystein hydrochloride hydrate 99%, 1,4-dithioerythritol, octafluoronaphthalene 96% (OFN) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) were from Aldrich (Steinheim, Germany), 2,3,4,5,6-pentaflourobenzyl bromide (PFBBr), 2-phenylethanethiol and 4-methoxy-α-toluenethiol were from Fluka. LiChrolut EN resins and 3 mL polypropylene cartridges were from Merck. Bond Elut ENV resins, pre-packed in a 50-mg cartridge (1 mL total volume) and a SPE VAC ELUT 20 station were from Varian (Walnut Creek, CA, USA). Pure water was obtained from a Milli-Q purification system (Millipore, USA).

Synthetic wine: water and ethanol 12%, 5 g L^{-1} tartaric acid and pH adjusted to 3.4

2.2. Wine samples

Eight monovarietal wines (red and rosé) made from Prieto Picudo (D.O. Tierra de León) and seven (red, rosé and white) from different grape varieties and six different Spanish denominations of origin. Details of the wines are expressed in Table 1.

2.3. GC-O analysis and identification of the target compound

2.3.1. Extract preparation

Wine volatiles were collected using a purge-and-trap system. The trap consisted of a standard polypropylene SPE (solid phase extraction) tube (0.8 cm internal diameter, 3 mL internal volume) packed with 400 mg of LiChrolut EN resin. Prior to analysis the bed was washed with 20 mL of dichloromethane and dried by passing air through it (negative pressure 0.6 bar, 10 min). The tube was placed on top of a flask containing 80 mL of wine. The temperature was 25 °C (room temperature). A controlled stream of nitrogen (500 mL min^{-1}) was introduced in the head space over the sample during 100 min. Then, the cartridge was dried by passing a small flow of nitrogen. The trapped volatiles were eluted with 3.2 mL dichloromethane with 5% methanol and the extract was concentrated under a stream of pure nitrogen to some 200 μL.

2.3.2. GC-O analysis

One microlitre of the extract was injected in splitless mode in a gas chromatograph Trace GC from ThermoQuest (Milan, Italy), equipped with a flame ionization detector (FID) and a sniffing port ODO-1 from SGE (Ringwood, Australia). The column was DB-WAX from J&W (Folsom, CA, USA), 30 m × 0.32 mm i.d., 0.5 mm film thickness. A constant pressure of 52 kPa of H_2 was applied throughout the analysis time. The temperature program was 40 °C for 5 min, then raised by 4 °C min^{-1} to 100 °C followed by 6 °C min^{-1} to 220 °C, and finally kept at 220 °C for 20 min. Injector and detector were both kept at 250 °C. To prevent condensation of high-boiling compounds at the sniffing port, the port was heated sequentially with a laboratory-made rheostat. A panel of six judges carried out the sniffings of the extract. Sniffing time was approximately 30 min and each judge performed one session per day. Panelists were asked to assess the overall intensity of each odor on a seven-point category scale (0, not detected; 1, weak, hardly recognizable odor; 2, clear, but not intense odor; and 3, intense odor; half values were allowed). A mixture of intensity and frequency of detection (denominated "modified frequency", MF) data were processed and calculated with the formula proposed by Dravnieks [16]:

$$\mathrm{MF}(\%) = \sqrt{F(\%) \times I(\%)} \quad (1)$$

where $F(\%)$ is the detection frequency of an aromatic stimulus expressed as a percentage and $I(\%)$ is the average intensity

Table 1
Wine samples and its characteristics. Quantitative data for 3-methyl-2-butene-1-thiol.

Wine	Kind of wine	Denomination of origin	Vintage year	Grape variety	Alcohol % (v/v)	MBT Conc.[a](ng L^{-1})
Tombú	Rosé	Tierra de León	2008	Prieto Picudo	13.5	0.6[b]
Peregrino	Rosé	Tierra de León	2009	Prieto Picudo	13	1.1[b]
Peregrino	Young red	Tierra de León	2008	Prieto Picudo	13	< 0.5
Pardevalles	Young red	Tierra de León	2008	Prieto Picudo	14	0.8[b]
Cumal	Aged red	Tierra de León	2005	Prieto Picudo	14	1.8[b]
Castillo de Valmadrigal	Aged red	Tierra de León	2005	Prieto Picudo	14	< 0.5
Leione	Aged red	Tierra de León	2005	Prieto Picudo	13.5	< 0.5
Don Suero	Aged red	Tierra de León	2000	Prieto Picudo	13	< 0.5
Viñas del Vero	Young red	Somontano	2008	Cab. Sauvignon/Merlot	13.5	< 0.5
Bodegas de Ávalos	Young red	Rioja	2008	Tempranillo	13.5	< 0.5
Muen	Rosé	Rioja	2010	*Unknown*	13.5	< 0.5
Care	Aged red	Cariñena	2005	Merlot/Syrah	13.5	< 0.5
Veliterra	White	Rueda	2010	Verdejo	13	< 0.5
Aragus	White	Campo de Borja	2008β	Chardonnay	13	< 0.5
Coto de Hayas	White (oak barrel fermentation)	Campo de Borja	2009	Chardonnay/Macabeo/ Moscatel	13	< 0.5

[a] Quantitative data of 3-methyl-2-butene-1-thiol.
[b] Estimated concentrations since the concentration was under the quantification limit.

expressed as percentage of the maximum intensity. Blank analyses were carried out by extracting a synthetic wine without aroma compounds and performing a complete olfactometric analysis. The identification of the odorants was carried out by comparison of their odors, chromatographic retention index in both DB-WAX and VF-5 columns and MS spectra with those of pure reference compounds.

2.3.3. Identification of the target compound by multidimensional gas chromatography (MDGC-MS).

Fifty microliters of the extract prepared as described in section 2.3.1 was injected in a multidimensional GC-GC–MS system from Varian (Walnut Creek, CA). The system consisted of two independent gas chromatographs interconnected by a thermoregulated transfer line kept at 200 °C equipped with a Deans valve switching system (Valco Instruments, Houston, TX), two olfactory ports, and FID and MS detectors, as described in Ref. [8]. Chromatograph 1 was equipped with a DB-Wax column (polyethylene glycol) from J&W (Folsom, CA), 30 m × 0.32 i.d. with 0.5 μm film thickness. Initially, the GC-O extract (50 μL) was monitored by olfactometry in the first chromatograph to select the fraction containing the target odorant. In further chromatographic runs, selective heart-cuttings were made to isolate the unknown odorant, which was transferred to the second chromatograph equipped with a FactorFour-VF-5 MS column (polymethylsiloxane-5% diphenyl) from Varian (30 m × 0.32 mm with 1 μm film thickness). In this second oven, isolated odorant was trapped in a CO_2 cryotrapping unit and monitored by olfactometry with simultaneous MS detection. The global run time was recorded in full-scan mode (m/z 45–250 mass range). The identity of the odorant was determined from the mass spectrum and linear retention indexes in both columns (DB-Wax and VF-5 MS) and confirmed by injection of the pure reference standard. Other technical characteristics of the equipment are described in a previous work [8].

2.4. Odor thresholds

Odor threshold was defined as the smaller concentration of the compound which can exert a significant effect (significance: 95%) on the aroma of wine. The sensory panel was composed of seven women and five men, 23–37 years old, all laboratory staff members with extensive experience in sensory analysis. Odor threshold of 3-methyl-2-butene-1-thiol was determined in several matrices: water, synthetic wine (water and ethanol 12%, 5 g L^{-1} tartaric acid, pH adjusted to 3.4), and rosé and red wines. For this purpose forced choice triangular tests [17] were performed for several concentration levels, starting with the highest one (20 ng L^{-1}) and successively being decreased by a factor of 2. In all cases, wine (20 mL at 20 °C) was served in coded, tulip-shaped glasses covered by glass Petri dishes and coded with a three digits number. Panelist had to decide which wine glass smelt different compared to the two others. Some descriptors of the perceived difference were requested. The number of correct answers was compared with tabulated values to decide if significant differences (95%) exist due to the added amount of the odorant. The odor threshold was considered as the geometric mean between the lowest concentration which provided a significant difference with respect to the reference wine and the highest concentration which did not supplied any difference.

2.5. Quantitative analysis

2.5.1. Method

Quantitative analysis of the identified compound was carried out using a method proposed earlier to analyze polyfunctional mercaptans [18]. In a 24 mL screw-capped vial, 23 mL of wine were spiked with 0.2 g of EDTA (5 g L^{-1}) and 0.6 g of L-cysteine chlorohydrate (0.1 M) and kept shaking for 2 min. After this, the wine was transferred to a 20 mL volumetric flask and spiked with 15 μL of an ethanolic solution containing 1400 μg L^{-1} of 2-phenylethanethiol as internal standard. Six milliliters of this sample were then loaded onto a 50 mg BondElut-ENV SPE cartridge (previously conditioned with 1 mL of dichloromethane, 1 mL of methanol, and 1 mL of water). Some wine major volatiles were removed by rinsing with 4 mL of a 40% methanol/water solution 0.2 M in phosphate buffer at pH 7.7 and, after this, with 1 mL of water. A second internal standard was added to the cartridge; 220 μL of an aqueous solution containing 15 μg L^{-1} of 4-methoxy-α-toluenethiol was loaded onto the cartridge. Mercaptans retained in the cartridge were directly derivatized by passing first 1 mL of an aqueous solution of DBU (6.7%) and later 50 μL of a 2000 mg L^{-1} solution of PFBBr in hexane, and letting the cartridge imbibed with the reagent for 20 min at room temperature (25 °C). Excess of reagent was removed by adding 100 μL of a 2000 mg L^{-1} solution of mercaptoglycerol in 6.7% DBU aqueous solution, and letting the cartridge react again for 20 min at room temperature. The cartridge was then rinsed with 4 mL of a 40% methanol/water solution 0.2 M in H_3PO_4 and with 1 mL of water. Derivatized analytes were finally eluted with 600 μL of n-hexane/diethyl ether (1/3; v/v), and then 10 μL of the chromatographic internal standard solution (octafluoronaphtalene, OFN, 22.5 μg L^{-1} in hexane) was added to the extract. The eluate was finally washed with five 1 mL volumes of brine (200 g L^{-1} NaCl water solution), transferred to a 2 mL vial, and spiked with a small amount of anhydrous sodium sulfate. Four microliters of this sample was directly injected in cold splitless mode into the PTV injection port and analyzed by GC–MS with negative chemical ionization (NCI) (more details are described in Ref. [18]). The analyte and internal standards ions were acquired in the single ion monitoring (SIM) mode: 3-methyl-2-butene-1-thiol is quantified with m/z 262 (263 and 213 as qualifier ions) and the quantification of the internal standards was carried out with m/z 135, 314 and 272 for 2-phenylethanethiol, 4-methoxy-α-toluenethiol and OFN, respectively.

2.5.2. Method validation

The validation study was carried out for three different potential internal standards: 2-phenylethanethiol, 4-methoxy-α-toluenethiol and OFN. Method linearity was studied by standard addition to one red, one rosé and one white wine. Five concentration levels between 1 and 200 ng L^{-1} were analyzed in duplicate. Precision was evaluated by the triplicated analysis of three different samples for every type of wine (white, rosé and red), spiked at two different concentration levels (low level: 10 ng L^{-1}; high level: 100 ng L^{-1}). Analyses were carried out on different days. In order to evaluate matrix effects, an experiment of standard recovery was carried out for nine wines (three reds, three rosés and three whites). These wines were spiked or not with known concentrations (50 ng L^{-1}) of the analyte. Recovery in synthetic wine was considered to be at 100%, since the calibration plot originally will be built using this matrix. A one-way ANOVA test was made to evaluate the possible existence of significant differences in recovery levels between sorts of wines. A t parameter was calculated to evaluate whether the average recovery for every internal standard was significantly different from 100% (significance level: 95%). Detection and quantification limits were defined as the concentration giving a peak height three or ten times the signal-to-noise ratio. During the analysis of real samples, wines spiked with the analyte (10 ng L^{-1}) and blanks were included in the batches to control the system.

Table 2
Odor zones detected in GC-O analysis of a Prieto Picudo wine ranked according to olfactometric intensity.

LRI (DB-Wax)	LRI (DB-5)	Odor description	Compound	% MF [a]
1217	719	Fusel, alcohol	Isoamyl alcohol	83
955	600/814	Lactic, strawberry	Diacetyl/ ethyl 2-Methylpropanoate	78
1067	856	Fruity anise	Ethyl 3-methylbutyrate	76
1149	846	Fruity, anise	Ethyl 2-methylbutyrate	75
1237	999	Sweet, fruity, anise	Ethyl hexanoate	69
1290	952/1004	Citric, solvent	Furfuryl ethyl ether/ octanal	69
1448	600	Vinegar	Acetic acid	66
1913	1108	Roses	β-phenylethanol	66
1032	800	Strawberry, lactic	Ethyl butyrate	65
1109	**805**	**Marihuana, rubber, beer**	**3-methyl-2-butene-thiol**	**61**
1125	860	Banana	Isoamyl acetate	53
1099	621	Fusel, rubber	2-methylpropanol	49
1620	1022	Burnt	2-acetylpyrazine	47
1952	1134	Coconut, wood	Z-whiskylactone	41
1768	–	Barbecue, roasty	¿?	37
1857	1086	Phenolic, medicine	guaiacol	37
1675	898	Cheese	3-methylbutyric acid	35
1010	906	Solvent, fusel	¿?	33
1419	1130	Fruity, sweet, anise	ethyl cyclohexanoate	33
1813	1392	Cooked apple, sweet	β-damascenone	31

Target odor zone is in bold type.

[a] Modified frequency calculated with expression (1).

3. Results and discussion

The GC-olfactometric study of a red wine revealed the existence of an unknown odor zone. Identification of the responsible compound or compounds was carried out and its occurrence and sensory role was studied.

3.1. GC-olfactometry

Volatile fraction of a red wine made from Prieto Picudo grapes (Cumal 2005) was isolated by means of a technique in which solid phase extraction (SPE) and head space analysis are combined. This extraction method allows the production of highly representative extracts for the GC-O analysis. Odorants and intensities perceived in olfactometry represent, with a high reliability, the real compounds and intensities noticed when a wine is smelled.

Table 2 shows the results of the olfactometry of the aged red wine in which the target compound was detected. As it can be seen, an odor zone with a retention index of 1109 in a polar column was described by the panelists as marihuana-like, rubber-like and beer-like. This zone reached a quite high modified frequency (61%). Only major compounds which constitute the base of the wine aroma like ethyl esters, fusel alcohols, acetic acid or diacetyl seemed to be more important in the aroma of this wine according to this set of data. Although other odor zones were not elucidated in this study, their modified frequency was not so high and the role in the aroma is likely less relevant. It should be noted that blank analyses were carried out in order to corroborate the analyte or analytes responsible for the odor came from the wine and assuring that it was not an artifact presents or formed during extraction or chromatographic process.

In a previous work carried out in our laboratory with several wines made from Prieto Picudo grapes [19] an odor zone with the same retention index and similar odor description was detected. However the identity of the responsible molecule or molecules was not elucidated. This odor zone presented a high modified frequency in some of the studied wines in that work.

According to these observations it was essential to find out the identity of this volatile compound or compounds, to study the role in wine aroma and to know if it is characteristic of wines made from Prieto Picudo grapes.

3.2. Identification of the target compound

A multidimensional gas chromatograph with mass spectrometry detection and olfactometry port was used to identify the target odorant or odorants. This system consisted of two coupled chromatographs with two columns of different phase. Both systems have an olfactometry port, thus the odorants could be detected by its odor. Firstly 50 μL of the SPE-HS extract used in the olfactometry study were injected and the odor zone was detected in the first dimension through the sniffing port. In further chromatographic runs, selective heart-cuttings (20 s length) were made to isolate that zone, which was transferred to the second oven and monitored by olfactometry with simultaneous MS detection. Several chromatographic peaks were observed in the second dimension but only one odor signal was detected in the olfactometry port. This means that only one compound was responsible for the perceived odor. This olfactometric signal corresponded with one of the peaks (Fig. 1). The identity of the odorant was determined from the mass spectrum (Fig. 1) and linear retention indices in both columns (DB-Wax, 1109; VF-5 MS, 805) and confirmed by injection of the pure reference standard. All data pointed out that the identity of the compound responsible for the odor zone was 3-methyl-2-butene-1-thiol (MBT). This molecule is commonly found in beer. It is known to be formed from precursors present in hops [20] and at low concentrations it seems to impart a pleasant hoppy flavor, while at higher concentrations, it is known to be responsible for the light struck off-flavor of lager beers [21,22]. This compound has been also detected in other matrices like coffee or essential oils of flowers [23–25]. With regard to wine this odorant is not a common component of its aroma however this is not the first time that this compound is detected in this type of beverage. Bailly et al. [26] tentatively identified MBT in Sauternes wine by GC-Olfactometry of a SPE extract. However in that case identification was not complete since mass spectrum was not provided and identification was based on odor and retention indices in two different columns. In fact most of the works in which this compound has been detected were based on olfactometry studies and no mass spectrum could be obtained. This observation shows the powerful odor this compound can exert. A formation pathway in wine has been proposed [26]. This compound may be formed

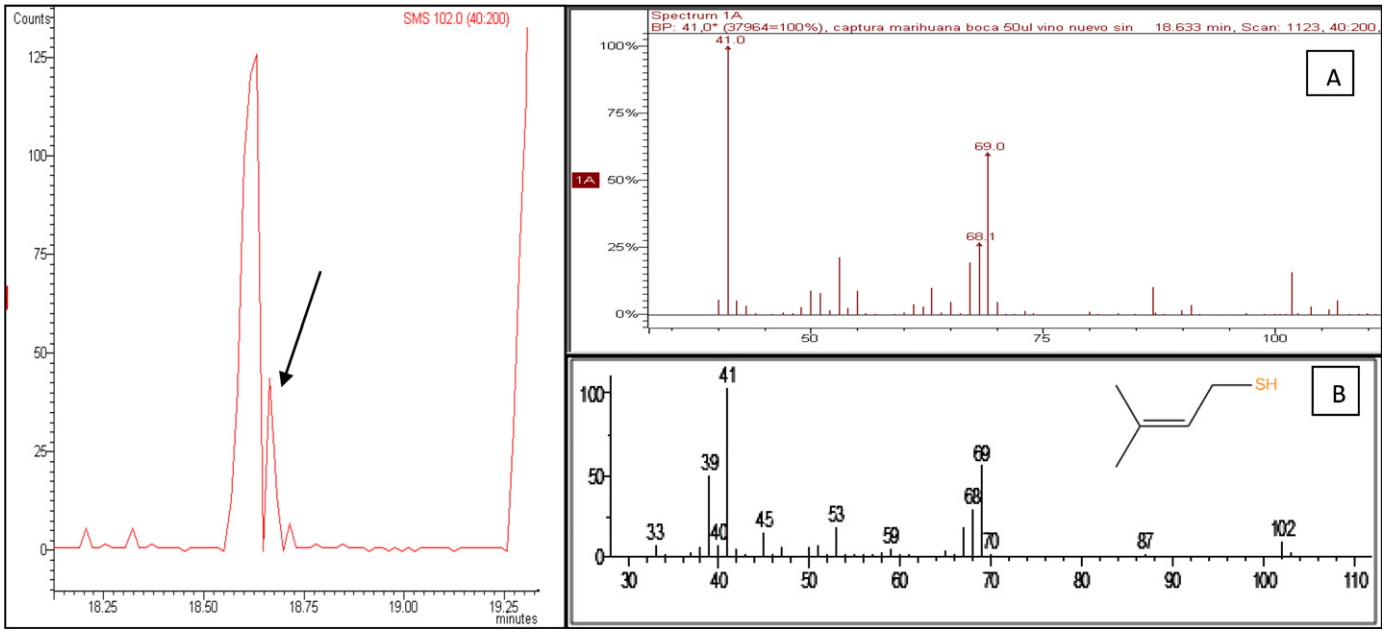

Fig. 1. Chromatographic peak (m/z 102) in GC × GC system and mass spectra of 3-methyl-2-butene-1-thiol. A, experimental spectrum obtained by GC-GC–MS; B, commercial standard spectrum.

from 3-methyl-2-buthen-1-ol by nucleophylic substitution by H_2S. The alcohol is formed by reduction of the corresponding aldehyde, which is found in must. This means that MBT content might be related to the grape variety.

An estimation of the concentration level was carried out by comparing to the MS-signal provided by an extract of a red wine in which a known amount of the analyte was added. According to that, the concentration of MBT was at about 2 ng L^{-1} in the studied wine. Furthermore, an extract of a wine in which that concentration was added provided a modified frequency in olfactometry of 60%, similar to that observed for the Prieto Picudo wine. These observations point out that the concentration of MTB in the targeted wine must be about 2 ng L^{-1}.

3.3. Odor threshold determination

In order to characterize the role that MBT has in wine aroma it is essential to know the minimum concentration of the odorant that has an effect on aroma perception. Therefore odor threshold was calculated in several matrices. First of all, threshold in water was found to be 0.01 ng L^{-1}, one of the lowest odor thresholds found in water. In synthetic wine, the threshold is 0.7 ng L^{-1} while in real wine it depends on the type of wine: 0.5 in rosé and 1 ng L^{-1} in red. These values are similar to the odor threshold found in beer for this compound, 2–7 ng L^{-1} [27]. It is worth mentioning that panelists pointed out a clear rubber odor when MBT was over 10 ng L^{-1} in red wine; which means that if the odorant is over that concentration it will be likely to cause an off-flavor and no contribution to positive nuances are expected. These sensory experiments corroborate the highly powerful odor this compound supply.

3.4. Quantitative method

Due to its potential importance MBT could have in wine aroma it would be interesting to know if this compound can be found in different wines and to determine its concentration. Therefore a highly sensitive method is necessary to the quantification of this analyte. For this reason an analytical procedure developed in our laboratory for the quantification of several polyfunctional thiols like 4-methyl-4-mercapto-2-pentanone at ng L^{-1} level [18] was checked for the analysis of MBT. In that method the analytes are isolated by a solid phase extraction procedure with derivatization into the cartridge by reaction between pentafluorobenzyl bromide and the –SH group. The corresponding derivatives are further eluted and determined by gas chromatography–negative ion mass spectrometry (GC–NCI-MS). This method provides a high selectivity and sensitivity in the analysis of thiols. In order to quantify MBT, the extraction procedure and chromatographic conditions were the same than those used in the analysis of the other thiols. Three internal standards were checked: 2-phenylethanethiol (method standard), 4-methoxy-α-toluenethiol (derivatization reaction standard) and OFN (injection standard).

Previous tests were carried out in order to check if this method allowed obtaining an appropriate chromatographyc signal for MBT. This was done by extracting synthetic wine containing different concentration levels of the analyte in a range between 1 ng L^{-1} and 1 μg L^{-1}. The extract was firstly analyzed by GC-NCI-MS in scan mode in order to know the mass spectrum of the derivatized compound (Fig. 2) and establish the mass which provided the highest sensitivity. The method allowed obtaining a considerable signal for MBT in synthetic wine at ng L^{-1} level. The ion with m/z 262 gives the highest signal-noise ratio (Fig. 2).

3.4.1. Method validation

Linearity. The linearity of the method was tested in white, rosé and red wine between 1 ng L^{-1} and 200 ng L^{-1}. Relative areas to an internal standard (2-phenylethanethiol, 4-methoxy-α-toluenethiol or OFN) were used in these tests. In all cases, for the three wines and the three internal standards, regression coefficients were greater than 0.99.

Precision. Precision was evaluated as repeatability by replicated analysis of spiked samples at two concentration levels: a low level: 10 ng L^{-1}, and a high level 100 ng L^{-1}. Relative standard deviations (%RSD) are expressed in Table 3. Excellent RSDs, lower than 10% in most of cases, were obtained in rosé and white wine for both addition levels and for the three internal standard. In case of red wine precision was acceptable for the lowest level of addition but it was poor for 100 ng L^{-1} spiked samples, above all using OFN as internal standard. This is due to

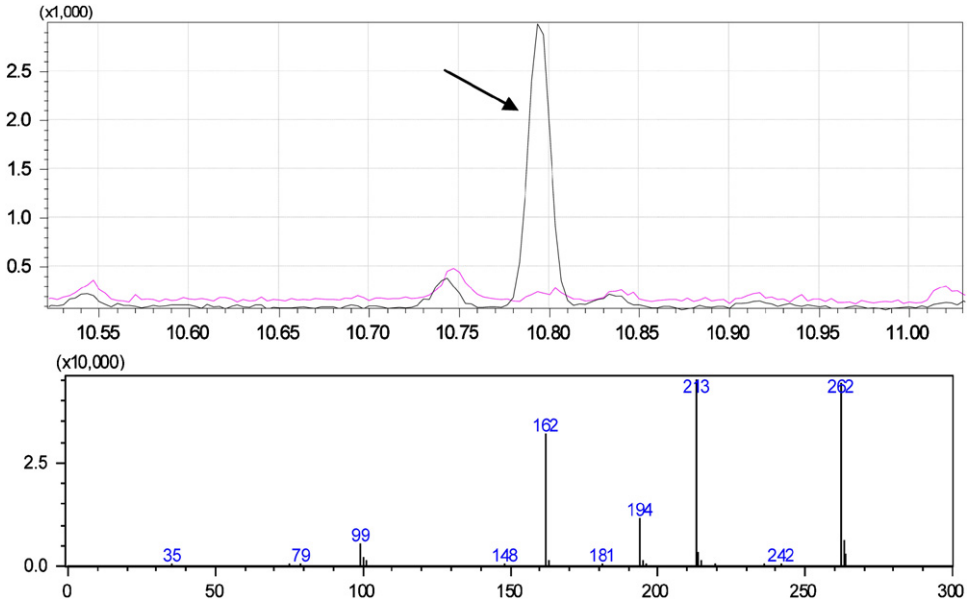

Fig. 2. Chromatographic peak (black chromatogram: 10 ng L^{-1} spiked to a synthetic wine) with *m*/*z* 262 and NCI mass spectrum of 3-methyl-2-butene-1-thiol derivatized by reaction with PFBBr.

Table 3
Repeatability of the method, expressed as RSD (%). Data are the average RSD (%) obtained in the replicated analysis of three different wines.

	Internal standard	Low level[a]	High level[b]
Red wine	OFN	13.8	32.6
	2-phenylethanethiol	10.8	17.8
	4-methoxy-α-tolulenthiol	14.3	18.5
Rose wine	OFN	11.3	5.5
	2-phenylethanethiol	12.3	8.0
	4-methoxy-α-tolulenthiol	7.6	4.2
White wine	OFN	8.5	7.2
	2-phenylethanethiol	2.4	6.7
	4-methoxy-α-tolulenthiol	9.4	9.6

[a] 10 ng L^{-1}.
[b] 100 ng L^{-1}.

the presence of a chromatographic interference in red wines close to the target peak when *m*/*z* 262 was considered. When the amount of analyte increased the tail of the interfering peak started to overlap the analyte's one and integration was less accurate. Quantification by using heights was considered however the precision for most of samples was much worse. Furthermore other *m*/*z* were tested but signal/noise ratios were low, thus sensitivity was poor. Therefore a relatively high uncertainty is expected when high concentrations (over 60 ng L^{-1}) are analyzed in red wines. In those cases the use of OFN as internal standard must be avoided. However such levels of concentration are unlikely to appear in wine.

Detection limits. Detection limits were calculated as the concentration that generated a signal of three times the signal–noise ratio, which were determined in spiked samples with low levels of 3-methyl-2-butene-1-thiol. 0.5 ng L^{-1} was the lowest concentration that can be detected by this analytical method. Quantification limit was 1.7 ng L^{-1}. This means that it is possible to detect concentrations of MBT around the odor threshold in wine but it is not possible to give quantitative data at this level. Therefore this sensitivity is slightly limited for studying the occurrence of the odorant when this will be at peri-threshold levels. Further improvements are needed to reach lower limits for this method.

Matrix effects. A standard recovery study was carried out by analyzing nine wines (three white, three rosé and three red wines). The increment in the signal obtained in the spiked wines was compared with the signal generated by the same amount of analyte added to a synthetic wine. The results are presented in Table 4. The data shown are the mean and standard deviation of the recoveries found in every kind of wine and a global average recovery was obtained for every internal standard. An ANOVA was made to evaluate the possible existence of significant differences in recovery levels between sort of wines. A t parameter was calculated to evaluate whether the average recovery was significantly different from 100%. Differences in recovery levels between sort of wines were only observed in case of OFN as internal standard. For 2-phenylethanethiol, a higher recovery was obtained for white wine but differences were not significant at 95%. In case of 4-methoxy-α-tolulenthiol any differences were observed. With regard to average recoveries, *t* parameters show that none of the internal standards corrected the matrix effects with respect to a synthetic wine, since mean recovery was in all cases significantly different from 100%. The highest values were obtained for 4-methoxy-α-tolulenthiol. Therefore, these observations suggest that calibration should be done in real wine with 4-methoxy-α-tolulenthiol as standard. This allows using the same calibration for every sort of wine and it provides good precision and linearity, as it has been previously commented.

This validation study has demonstrated that it is possible to quantify the target molecule, 3-methyl-2-butene-thiol by an existing method in the concentration levels that are able to exert a sensory effect on wine aroma.

3.5. Occurrence of MBT in wines

Fifteen samples were analyzed in order to study the occurrence of the analyte and its possible link with Prieto Picudo wines. Eight of these wines were made from Prieto Picudo grapes and the rest were from different grape varieties and production zones. Quantitative data are shown in Table 1. As can be seen, the compound has been detected in four of the 15 analyzed wines. These four samples were made from Prieto Picudo grapes which points out that 3-methyl-2-butene-1-thiol can be an odorant related to the variety. The wine in which this compound was detected by GC-O was also analyzed and a concentration of

Table 4
Average recoveries with their standard deviation and statistical tests for checking matrix effects. Results are the average of the recoveries found in the analysis of three red, three rosé and three white wines. Recovery in synthetic wine is considered 100%. Significant differences (95%) are shown in bold.

Internal standard	Red wine % recovery	SD[a]	Rose wine % recovery	SD	White wine % recovery	SD	%R mean	SD	ANOVA[b]	t_{exp}[c]	$t(95)$[d]
OFN	**11.7**	2.5	**28.4**	7.6	**45.8**	2.3	28.6	17.0	<0.001		
2-phenylethanethiol	65.1	7.1	67.5	7.1	78.6	3.8	70.4	7.2	0.079	**7.1**	2.92
4-methoxy-α-tolulenthiol	80.0	9.9	83.7	6.3	81.4	11.1	81.7	1.8	0.891	**17.2**	2.92

[a] Standard deviation.
[b] Significance of ANOVA test.
[c] t experimental value (95% significance) for the comparison of the average percentage of recovery versus 100%.
[d] t critical parameter value (95% significance) for the comparison of the average percentage of recovery versus 100%.

1.8 ng L^{-1} was found. This piece of data corroborates estimations made by MDGC-MS. Two rosés and one young red were the other wines in which the analyte was detected, at concentrations around the odor threshold. At this level the sensory effect was not clearly perceived. When panelists compared rosé wines with added concentrations of 0.5–1 ng L^{-1} and reds with 1–2 ng L^{-1} to wines with no analyte content, slightly aromatic differences were described. Some positive descriptors like herbal or fresh were provided but negative contributions were also mentioned, for instance, a lesser fruity of aroma. A consensus of the real aromatic contribution of 3-methyl-2-butene-1-thiol presents at those levels on wine aroma was not achieved

In conclusion 3-methyl-2-butene-1-thiol, an almost unknown odorant in wine, has been detected in several Prieto Picudo wines. However at concentrations in which the analyte has been found, the aromatic contribution is subtle and not clearly described.

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