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Gas Chromatographic-Sulfur Chemiluminescent Detector procedures for the simultaneous determination of free forms of volatile sulfur compounds including sulfur dioxide and for the determination of their metal-complexed forms

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Highlights

- Free SO_2 and free VSCs simultaneously determined in the wine headspace at 30°C
- Best detection limits (LDs) with headspace-cryotrapping-GC-SCD. No SPME required
- LDs of 3 (H_2S), <100 (CS_2, MeSH, EtSH, DMS, DES, DEDS), <500 ng/L (molecular SO_2)
- Linearity holds complete range of occurrence. Precision satisfactory
- Metal-complexed forms determined in the headspace of 1:10 brine dilution at 70°C

Abstract

Three different procedures for the quantitative assessment of free and metal complexed volatile sulfur compounds (VSCs) and for the determination of truly free SO_2 have been developed, taking advantage of a GC-sulfur chemiluminescent detector system (GC-SCD) with cryotrapping. The inertness of the inlet systems, together with the column used (SPB-1 sulfur) makes it possible to obtain a non-saturated perfectly Gaussian peak for SO_2, well resolved from H_2S. In the main procedure, the injection of 1 mL of the headspace of a sample prepared in complete anoxia and equilibrated at 30°C makes it possible to get highly sensitive signals for all VSCs and free SO_2. Detection limits are 3, 35 and 60 ng/L for H_2S, MeSH and EtSH, 13 μg/L for truly free SO_2 (at pH=3.4, or 0.46 μg/L for molecular SO_2), and better than 1 μg/L for other relevant sulfur volatiles. Method precision is also satisfactory and linearity covers the whole range of occurrence of these compounds. A second procedure, not making use of the cryotrapping unit, gives also satisfactory results, although with higher detection limits (0.03, 0.25 and 0.37 μg/L for free H_2S, MeSH and EtSH, respectively). For the analysis of free plus metal-complexed forms, it has been demonstrated that the headspace injection of the vapors on a 1:10 brine dilution of the sample heated at 70°C for 25 min, gives good estimates of the free + metal-complexed forms of H_2S and wine mercaptans.

Keywords: hydrogen sulfide, mercaptans, copper, reduction, off-odors, metal-complex

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1. Introduction

The so called volatile sulfur compounds (VSCs) are a group of small odor molecules containing at least a sulfur atom. Some of these molecules may have a strong impact in the odor profiles of many fermented products and their control represents an active challenge for the food industry. The more important from the aromatic point of view are hydrogen sulfide (H_2S) which smells of rotten eggs, methanethiol (MeSH) with a rotten cabbage odor, ethanethiol (EtSH) with cooked cabbage and onion odors, and dimethylsulfide (DMS) with a characteristic asparagus and truffle odor. H_2S, MeSH and EtSH are the principal responsible for an important wine off-odor known as “reductive odor” [1-3]. DMS, on the contrary, may have a positive effect enhancing fruity notes depending on its concentration [4, 5].

The odorants involved in the reductive odor are mainly formed during fermentation. Yeast transforms sulfate and sulfite into sulfide through an enzymatic chain known as sulfate reduction sequence (SRS) [6, 7] in order to produce cysteine and methionine [8]. MeSH can be formed from methionine [9] and its intracellular esterification can produce methyl thioacetate. EtSH may be formed by the reaction between H_2S with ethanol or acetaldehyde and its corresponding thioacetate is similarly formed by intracellular esterification. These thioesters have odor thresholds 15-40 times higher than MeSH and EtSH, meaning that it is unlikely that these compounds may be direct responsible for off-odors, but can hydrolyze at wine pH regenerating MeSH and EtSH during wine aging [10]. The oxidation product of MeSH, dimethyldisulfide (DMDS), can be also found in wines. On its side, DMS is mostly formed by slow hydrolysis of its precursor, the grape *S*-methyl methionine [11].

H_2S and mercaptans in wine can exist under three different categories of chemical species interconnected via chemical equilibria: free forms, metal-complexed forms and mixed disulfides and polysulfanes, some of which act as oxidized precursors [12, 13]. Only free forms are odor active. They are in equilibrium with metal complexed forms, which are mainly

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constituted by complexes with Fe, Cu and in the case of H_2S, also with Zn [14]. Oxidized precursors are poorly known, although recent studies suggest that they are mainly mixed disulfides with wine cysteine and glutathione and in the case of H_2S, different polysulfanes [15]. The existence of these types of species and of their associated chemical equilibria explains the persistence of reductive problems and the long term inefficiency of the most common remedial actions: copper treatments and micro-oxygenation [12, 16, 17]. Oxidized precursors are at present determined using the same methods used for free or metal-complexed forms after a reduction process [15, 18].

The main analytical technique for the determination of VSCs is gas chromatography with sulfur-selective detectors. One of the most used is the pulsed flame photometric detector (pFPD) because of its sensitivity and ease of use [19, 20]. The major drawback of this detector is the quadratic response for sulfur compounds [21], which in practice implies quite limited dynamic working ranges, meaning that most concentrated samples have to be diluted and re-analyzed. The sulfur chemiluminescent detector (SCD) has a relatively similar sensitivity but its response is linear and equimolar for all sulfur-containing molecules and dynamic ranges expand for several orders of magnitude [22]. The chemiluminescent reaction in which this detector is based is, a priori, less prone to matrix-derived interferences [22, 23]. This detector has been widely used in the petroleum and food industries [24, 25]. However, the robustness of early designs has been repeatedly questioned due to irreproducibility in the burners. Attending to the manufacturer, those problems have been solved in the last version of this instrument [26].

The sensitivities of pFPD and SCD detectors are high enough so that sample preconcentration requisites are not very strong. In spite of that, early designs required the injection of large volumes of headspace gas [23, 27], relatively high sampling temperatures [14] or salting out effects [2, 28]. Alternatively, many authors have used solid phase microextraction (SPME) as

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preconcentration strategy [28-33]. The matrix effects associated to competence towards active adsorption sites in the fiber [34] have been circumvented by dilution with brine [32], by the use of standard addition as calibration method [28] or by using deuterated isotopomers as internal standards [33]. Nevertheless, the SPME of these components can induce the formation of artifacts [35, 36] and in any case requires a careful calibration of each fiber.

However, the existence of different chemical species in equilibrium, makes that the sample treatment should be consistent with the species to be determined. In the case of free forms, any change in the sample conditions affecting to the chemical equilibria should be avoided. In the case of wine, this implies that salting out effects should not be used, that SPME preconcentration should be carefully assessed and that the analysis should be done at temperatures as close as possible to normal consumption temperatures.

Moreover, there is no reason at present to not include the "truly" free SO_2 [37] as a target analyte in the routine determination of free VSCs. The International Organization of Vine and Wine (OIV) recommends the aeration-oxidation method (A-O) for determining sulfur dioxide. This method, however, overestimates the levels of free SO_2 of the wine, since when wine is acidified, some labile complexes between this molecule and anthocyanins and other phenols cleave. As those labile complexes may affect to the effective properties of SO_2 [37], it is convenient to develop methods able to measure SO_2 without wine acidification. A simple production-oriented method recently developed by Coelho et al. [38] makes use of headspace gas detection tubes (HS-GDT), and a sensitive (LOD of truly free SO_2 0.5 mg/L) and accurate instrumental method based on HS-GCMS has been also recently proposed [37].

In the case of metal-complexed forms of thiols, there has been some controversy about the best procedure for cleaving complexes. Previous reports proposed a 1:25 dilution in brine for determining Brine Releasable VSCs (BR-VSCs) [14], a most recent work suggest that a 1:10

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dilution is enough [39], while other authors assume that simple NaCl addition is enough to cleave complexes [40].

Because of all these reasons and taking advantage of the sensitivity of the new design of the commercial SCD detector, the goals of the present paper are to develop a series of analytical strategies able to provide 1) a sensitive and accurate determination of free forms of VSCs, including also free SO_2; and 2) a reliable determination of metal-complexed forms of H_2S and mercaptans not requiring SPME preconcentration.

2. Materials and methods

2.1 Reagents, standards and solvents

DMS was obtained from Merck (Darmstadt, Germany). DMDS, EMS, DES, CS_2, DEDS, MeSAc and EtSAc were supplied by Sigma-Aldrich (Steinheim, Germany). Sodium sulfide Na_2S, sodium methanethiolate CH_3SNa and sodium ethanethiolate CH_3CH_2SNa (also from Sigma-Aldrich) were dissolved in water at pH 9.6 for preparing H_2S, MeSH and EtSH. These solutions were daily prepared and were kept in the anoxic chamber. Sodium metabisulfite ($Na_2S_2O_5$) was purchased from Panreac (Barcelona, Spain). All the solutions and samples were prepared in the interior of a glove box P[BOX] (Jacomex) and they were stored out the glove box at -20°C, following recommendations of previous works [12, 14].

Brine contained 350 g/L of NaCl (from Panreac, ARG quality, Barcelona, Spain) in Milli-Q water (Millipore, Billerica, MA, USA). Synthetic wine was a pure water solution containing 12% v/v ethanol (gradient grade for liquid chromatography, Merck), 5 g/L of tartaric acid and pH adjusted at 3.4 with an aqueous solution of NaOH (0.1 M).

Metal salts were $CuSO_4 \cdot 5H_2O$, $Fe(NH_4)_2(SO_4)_2 \cdot 6H_20$ and $ZnCl_2$ from Panreac, ARG quality.

2.2 Wines

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Different Spanish wines were used to study the repeatability of the developed methods: a wine made with Chardonnay from La Mancha in 2015, a wine made with Grenache from Cariñena in 2001, a wine was from 2014 made with Mencía in Bierzo (León) and two wines made with Tempranillo, one of them in Ribera del Duero and the other in La Rioja from 2016 and 2017 respectively. This Tempranillo and a commercial wine (El Coto, 2016 from La Rioja) were used during the optimization of the analysis of Br-VSCs.

2.3 Simultaneous analysis of free VSCs and real free SO_2

The wine was opened within the anoxic chamber and 12 mL were transferred to a 20 mL standard headspace vial. Then 40 µL of the “A” internal standard solution (10 mg/L EMS in ethanol) were added, the vial was closed, taken out from the glove box and was immediately put in the sampler tray. The vial was incubated and its headspace sampled and injected under the conditions described in Table 1.

2.4. Analysis of Br-VSCs

The wine was opened within the glove box and 1.2 mL were transferred into a 20 mL standard headspace vial already containing 10.8 mL of brine. Then 40 µL of the “B” internal standard solution (2 mg/L EMS in ethanol) were added, the vial was closed, taken out from the glove box and was immediately put in the sampler tray. The vial was incubated and its headspace sampled and injected under the conditions described in Table 1.

2.5. Gas chromatography

Analyses were carried out using an Agilent 7890B gas chromatograph with a selective detector SCD 8355. The capillary column was a SPB-1 SULFUR (30 m x 0.32 mm I.D. x 4 µm film thickness (Supelco, Bellefonte PA, USA) preceded by a precolumn, 3 m x 0.32 mm I.D. of fused silica with

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a polar deactivation. The precolumn crosses inside the Cryogenic Trapping System (CTS 2, Gerstel).

The injection was made into a MMI injector equipped with an ultra-inert liner of 1 mm I.D. when cryofocusing was used and with an ultra-inert liner of 4 mm i.d. if cryofocusing was not used, both of them from Agilent. The autosampler was a Combi-PAL from CTCAnalytics (Zwingen, Switzerland) with a static headspace unit. After the injection, the syringe was purged with nitrogen for 5 min.

The chromatographic oven was held at 35 °C for 3 min (3.8 min if cryofocusing was used) then heated to 160 °C at 10 °C/min and held at this temperature for 0.5 min. Helium was used as carrier gas at a constant flow rate of 2 mL/min if cryofocusing was used. If not, then the He flow is set first at 0.9 mL/min for 0.8 min and then at 1.4 mL/min for the rest of the program. The temperature program of CTS 2 was as follows: -150 °C for 0.8 min and then raised at 20 °C/s up to 300°C. Air was used at 50 mL/min as oxidizer gas for the detector, base temperature and burner temperature were 280 °C and 800 °C, respectively. Hydrogen was used also for the detector, the upper flow was 38 mL/min and the lower flow was 7 mL/min.

2.6 Quantification of compounds

Calibration curves were built by adding known amounts of the sulfur compounds to model wine, which was analyzed as previously described for free or for BR-forms. Each point in the calibration plot was analyzed in duplicate. In all cases, areas normalized to the internal standard were used. Concentrations were estimated by interpolation of the measured signals in the calibration curves.

In the case of SO_2, the concentration of this component in the model wine was expressed in specifically SO_2 species (SO_2(gas)+SO_2(dissolved)+H_2SO_3), or “molecular SO_2”, using the expression:

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$$|SO_2| = C_{SO2} \cdot \frac{|H^+|}{|H^+| + K_a}$$

where $|SO_2|$ is the molecular SO_2 in mg/L, C_{SO2} are the mg/L of SO_2 of all SO_2 species present in the solution (basically SO_2 species and HSO_3^-), or "free SO_2", $|H^+|$ is the concentration of protons at the pH of the solution and K_a is the 1st acidity constant for H_2SO_3, which in hydroalcoholic solution was estimated to be 0,01096 [38]. Then, the interpolation of the signal obtained for SO_2 in the analysis of sample "i" gives directly its molecular SO_2, or $|SO_2|_i$ which is transformed into truly free SO_2 (in mg/L) attending to:

$$C_{truly\ free\ SO2i} = |SO_2|_i \cdot \frac{|H^+|_i + K_a}{|H^+|_i}$$

where $|H^+|_i$ is the concentration of protons at the pH of wine i.

The limit of detection (LOD) was defined as the concentration which gives a signal to noise ratio of 3. These ratios were calculated with the lowest point of the calibration curve for each compound.

2.7. Conditions for optimal release of metal-complexes VSCs

A number of studies were carried out in this section. The first parameters to optimize were the incubation times and temperatures to obtain maxima signals of the analytes. For this, both real wines and synthetic wines containing only analytes, were incubated at different temperatures (between 35 and 70°C) and times (between 5 and 60 minutes). In all these experiences, the samples were diluted 1:25 with brine, as in the previous method [14]. The second parameter assessed was the level and type of dilution. This study was carried out with different wines with reductive-odor problems. Samples were spiked with NaCl (2 g) or diluted with brine at different levels (1:4 and 1:10). Other conditions considered the addition of HCl to the brine, or the dilution of the wine with water (1:2 or 1:4) followed by NaCl addition (2g). In all cases analyses were carried out in triplicate (Results are shown in supplementary material). In the

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final set of experiments, different wines were spiked or not with metals and were then analyzed using three different conditions (NaCl addition and dilutions in brine 1:4 and 1:10) to determine which one was most efficient at cleaving the complexes. Metals spiked were Cu^{2+}, Cu^{+}, Zn^{2+} (all spiked at 500 µg/L) and Fe^{2+} (spiked at 1 mg/L). Spiked wines were kept with the metals in anoxia different times before the analysis (between 1 hour and 1 week). All these experiments were carried out in triplicate.

3. Results and discussion

This paper summarizes several studies related to the analytical determination of free and bound forms of VSCs using a SCD-detector. Truly free SO_2 is also determined in the run for free forms.

3.1 Analysis of free forms

3.1.1 Method development

The selected column was a SPB-1 sulfur (30 m x 0.32 mm x 4 µm) whose low bleed guarantees a good detector performance and a longer life for the ceramic burner. Additionally, this column makes it possible to get very good peaks even for SO_2, which in other more polar or less deactivated phases, elutes as a highly distorted and broad peak, rending its quantification impossible. The peak obtained for SO_2 in this particular GC system, which includes a highly deactivated inlet, is better in shape than the one we obtained in a GC-MS system with a standard split/splitless injector. As mentioned in the introduction, this makes unnecessary the addition of aldehydes to the wine. A typical chromatogram, obtained without cryofocusing can be seen in Figure 1. As can be seen, both H_2S and SO_2 elute well separated and with narrow and symmetrical peaks, which makes it possible to quantify both.

Two different analytical procedures, involving or not cryofocusing at the column head, were developed for the analysis of free forms. Injection conditions for both procedures are

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summarized in Table 1. In both cases, sample volume was 12 mL, the maximum for a safe headspace injection, and the incubation temperature was set at 30°, the minimum to ensure a good temperature stabilization. This temperature should be kept as close as possible to room temperature in order to mimic wine consumption and in order also to avoid potential equilibrium shifts from metal-complexed forms to free forms, which could cause some bias. In some of the previous methods, incubation temperature was set as high as 60°C in order to obtain the required sensitivity. In this system, however, even without cryofocusing, the sensitivity obtained at 30°C is highly satisfactory.

Obviously, if cryofocusing is not used, the injection has to be carried out in split mode to get narrow peaks for the poorly retained H_2S and SO_2. As seen in table 2 and in figure 1, a 1:20 split and a fast 1 s injection of 1 mL of headspace produced good chromatograms, with sensitivities better than those obtained in the pFPD system. In these conditions LODs achieved for SO_2 are highly satisfactory, much better than that previously reported by Carrascon et al. [37] (0.5 mg/L). However, in these conditions the sensitivities obtained for the two mercaptans, MeSH and EtSH, are not good enough, since their odor thresholds are very low and concentrations in the 0.5 ppb range should be easily quantifiable in order to obtain a reliable estimation of the role of these compounds in wine aroma. Because of this, the analysis of free forms should be preferably carried out with cryofocusing.

In order to obtain satisfactory chromatograms using cryofocusing, it was necessary to use as precolumn a fused silica capillary with a polar deactivant, since if the unavoidable condensation of some water and ethanol takes place in the non-polar SPB-1 sulfur column, the first peaks elute distorted. As for the trapping temperature, it was necessary to cold the trap until the minimum temperature (-150°C) in order to keep H_2S and SO_2 eluting as narrow separated bands. Another parameter to optimize was the rate at which the sample headspace is injected. Too higher rates may overload the system causing different effects, including

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insufficient cryofocalization and even the contamination of anterior parts of the inlet. On the other side, too slow injection rates may also overcome the retention ability of the cryofocusing unit. In our conditions, injection rates of 50 μL/s or higher were too high, and of 20 μL/s or smaller, too small. Accordingly, 30 μL/s was used as a compromise for the injection of 1 mL of headspace using a 1:2 split ratio, as summarized in Table 1.

Under these conditions, the improvement in sensitivities were in some cases very close to the maximum expected of 1 order of magnitude, such as for H_2S, MeSH or DMS, suggesting an optimum performance of the cryofocusing operation. Remarkably, detection limits for MeSH and EtSH are well below 0.1 μg/L, which should make it possible to make for the first time a realistic assessment of the potential implication of these two molecules, particularly EtSH, in reductive off-odors.

It should be noted that the method detection limits are better than those reported by Siebert et al. in 2010 using a previous version of the SCD (355 model SCD instead of an 8355 SCD) and relatively similar sampling conditions. Results reported here are particularly better for H2S, for which detection limits are more than 1 order of magnitude smaller. This improvement cannot be entirely attributed to the new instrument, but to improvements in the inertness of the liners and columns and to the strict anoxic conditions in which samples are prepared in our procedure.

3.1.2 Method validation

Method precision was studied for both procedures, with and without cryofocusing, by replicate analysis (n=3 or 4) of five different wines. Results are summarized in Table 3.

As can be seen in the table, the repeatability for the method without cryofocusing is in general acceptable, but it is not enough in some cases for an accurate determination of VSCs at low levels. As aforementioned, the most evident case is that of ethanethiol, which could be

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detected with this method only in one sample containing 1.6 μg/L. Some less important aroma compounds, such as DMDS, CS_2, MeSAc or EtSAc, could not be detected in most samples with this procedure. Imprecision in the low concentration range for H_2S and MeSH was also higher than desirable. The improvement in precision obtained with the cryofocusing unit is particularly noticeable in the quantification of low ranges of H_2S and MeSH, for which satisfactory repeatabilities were obtained. The determination of EtSH was not as precise, but yet acceptable values even for concentrations of just 0.4 μg/L were obtained. Other VSCs, such as DMDS, CS_2 or EtSAc, could be detected only with the method using cryofocusing. Although these compounds are not aromatically very important, their determination may be relevant since the acetates can be precursors of MeSH and EtSH, and DMDS can provide information related to the redox status of the wine. The determination of SO_2 is also satisfactory, although in this specific case, best results were obtained without cryofocusing.

The linearity of both procedures was studied with model wines. Results of the study are summarized in Table 4. As can be seen, in both cases linearity was satisfactory and no significant deviation of linearity was observed in the working ranges. These ranges cover the normal ranges of occurrence of these compounds in wine. It should be noted that calibration ranges cover in some cases more than 2 orders of magnitude, which represents a clear advantage in comparison with the working ranges attainable with the pFPD, which in most cases were below 1 order of magnitude.

3.2 Analysis of brine-releasable forms of VSCs (BR-VSCs)

Brine releasable forms of VSCs include free forms and metal-complexed forms. Dilution in brine was first proposed in 2007 as a way to solve the matrix effects observed in the headspace SPME analysis of VSCs [32]. Later, in 2014, it was demonstrated that those matrix effects were due to the presence of cation metals (Cu^{2+}, Fe^{2+} and Zn^{2+}) with which H_2S and mercaptans are known to form stable complexes. In those previous studies, the quantification

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of this fraction required a SPME preconcentration to account for the dilution (1:25). Taking advantage of the slightly higher sensitivity of the SCD detector, our goal was to develop a direct headspace method for the analysis of this fraction. Avoiding the use of SPME could be advantageous since SPME fibers and their batch to batch variability can be an additional source of uncertainty and in any case, requires changing the configuration of the automated sampler after or before the analysis of free forms. In order to compensate for the loss of sensitivity, the incubation temperature has to be increased. The optimization of this procedure is addressed in the following paragraphs.

3.2.1 Method optimization

In a first set of trials, carried out both with real and model wines (always diluted 1:25 in brine), different sample incubation times and temperatures were studied. In model wines, temperature affected mainly to the signal of H_2S, which increased (ca. 25%) when temperature was increased from 40 to 70°C (supplementary material). In real wines the effects were more marked and were wine-dependent. In the wine shown in Figure 2a, signals at 70°C were 5 times higher than those obtained at 35°C, while the signals obtained at 60°C were just 10-25% smaller than those obtained at 70°C. However, in the wine shown in Figure 2b, the signals at 70°C are more than 2 times above those obtained at 60°C. Differences between model and real wines and between real wines should be related to the different types of structures and interactions in which H_2S is involved in each wine. Recent evidences suggest that in the particular case of copper, nanoparticles of different sizes can be formed [41], and that the fraction of H_2S in free forms depends on the wine redox potential [12, 13]. Regarding incubation times, at 70°C the maxima signals were in all cases obtained after 15-25 minutes, and were stable at least up to 35 min (data not shown). As suggested by the continuous increasing trends observed with time for the areas of H_2S at 35 and 60°C, the equilibrium at 60°C and smaller temperatures seems to be attained at quite larger times, which suggests that

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at these temperatures the release of the VSCs from their complexes with metals takes more time. Because of all these results, a temperature of 70°C and an incubation time of 25 minutes were selected as optimum. At such temperature cryofocusing is not advantageous since the headspace is much enriched in water and ethanol, which strongly limits the volume of headspace that can be introduced into the column. The optimal sampling conditions are summarized in Table 1.

The other parameter which was further considered is dilution. In the original work, wine was diluted with brine 1:25. This level of dilution may be required to avoid matrix effects in SPME, but it is too large and may provide too high detection limits for some compounds. Other authors have recently demonstrated that 1:10 dilution is enough to have a good release from metals [39]. On the contrary, some reports use direct addition of NaCl [40].

First, dilutions of wine with brine 1/10 and 1/25 were compared. Studies were carried out with two different wines. The signals obtained per volume of wine were not significantly different regardless of the dilution level, both for H_2S and MeSH in both wines (Supplementary material). Such results confirm that 1/10 dilution is enough for cleaving complexes, in accordance to [39]. A second study comparing the direct addition of salt with dilutions with brine 1:4 or 1:10 was further carried out with four different wines: two commercial bottled wines and two wines directly taken from aging vats in the cellar. Results for H_2S are summarized in Figure 3, revealing a strong dependence on the wine type. In the two wines directly taken from the cellar the signals for H_2S (normalized by IS and sample volume) were independent on the dilution, suggesting that complex cleavage had been equally efficient in the three conditions. However, in the two bottled wines, the signals strongly and significantly increased with dilution, suggesting that the cleavage of complexes was strongly dependent on the dilution level in brine. At this point, it was not really clear why different wines behave differently, although it can be hypothesized that bottled wines have been stored in anoxic

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conditions for long time, so that H_2S-metal complexes may have form longer structures making complex cleavage more difficult [12, 42].

This question was further examined by widening the range of wines compared and also by spiking some wines with salts of Cu, Fe and Zn. Results of the first experiment (data not shown), revealed that in general, no dilution or small dilutions with brine produced far more imprecise signals for H_2S. In addition, in some particular wines, the signals were also significantly smaller than those obtained at 1/10 or 1/25 dilutions with brine. In the experiments carried out adding different cations to wines, one first relevant conclusion is that results depended on the time that the cations were left to equilibrate in wine. In the case of Cu(I), recoveries were quantitative only if the analysis is carried out immediately after the addition of the copper; after 3 hours, recoveries were significantly smaller except for the 1:10 and 1:25 dilutions (data not shown). In the case of Fe^{2+} and Zn^{2+}, the slow formation of their complexes with H_2S had already been observed in 2014 [14]. In these cases, aliquots of a wine with clear reductive problems were spiked with salts of Zn^{2+} and Fe^{2+} at normal wine levels, and were kept for one week in anoxia. Afterwards, the spiked wines were analyzed using different dilutions with brine. Results are given in Figure 4.

There are several relevant observations from data of this experiment. First, the imprecision of the signals significantly decreases with the level of dilution. The averaged relative standard deviation obtained by direct addition of salt is significantly higher (F test, significant at $P<0.05$) than that obtained at 1:10 dilution. Second, the signal significantly decreases with the presence of Zn^{2+} in the first two conditions. In both of them, the signals obtained in the presence of Fe^{2+} were smaller than those from the controls, but differences were not significant. However, in the presence of Zn^{2+} the H_2S signals were significantly reduced a 48% (first condition) or 32% (second condition). This implies that the addition of NaCl and the 1:4 dilution with brine are not able to break the complexes between H_2S and Zn^{2+}. The experiment

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also shows that the 1:10 dilution with brine provides signals whose magnitude is not significantly affected by the presence of those cations, suggesting that complex cleavage has been satisfactory in this condition.

3.2.2 Method validation

The analytical characteristics of the method are summarized in Table 5. Method precision was determined by replicated analysis (n=3 or 4) of six different wines. As can be seen in Table 5, the precision of the determination of BR-H_2S is not very high. In general, the repeatability obtained is better than 10% for most samples, but in some particular samples, not clearly assigned to a specific category or to a concentration level, consistent values close to 15% or even higher can be obtained. This sample effect had been already noticed with the previous method using SPME, which suggests that it is related to the nature of the complexes formed in some wines. In the case of MeSH, the average repeatability was 15.6% for samples with levels close to the quantitation limit, and it was around 7.5% for normal samples. In the case of EtSH, only 1 out of the 6 samples had quantifiable levels. Detection and quantitation limits were satisfactory in the case of H_2S and adequate for MeSH and EtSH, taking into account that the method assess BR-forms. The linearity of the method was also satisfactory and hold along the whole range of occurrence of the analytes.

4. Conclusions

Three different procedures for the quantitative determination of free and metal-complexed forms of VSCs in wine have been developed. The use of a highly inert inlets and chromatographic column combined with a cryofocusing unit, and with the preparation of samples in strict anoxic conditions, make it possible the highly sensitive simultaneous determination of truly free SO_2 and of free VSCs in the same HS-GC-SCD run. Detection limits obtained for MeSH and EtSH are low enough to make for the first time a reliable assessment of their sensory roles.

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It has been also demonstrated that the static headspace analysis of a 1:10 brine dilution of the sample incubated at 70ºC for 25 minutes, provides a good estimate of the free+metal complexed forms of H_2S and wine mercaptans, while smaller dilutions may fail in cleaving all the complexes between H_2S and Cu(I) and Zn(II).

5.Acknowledgements

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6. Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version

7. Bibliography

[1] O.J. Goniak, A.C. Noble, Sensory study of selected volatile sulfur-compounds in white wine, American Journal of Enology and Viticulture, 38 (1987) 223-227.

[2] T.E. Siebert, M.R. Solomon, A.P. Pollnitz, D.W. Jeffery, Selective Determination of Volatile Sulfur Compounds in Wine by Gas Chromatography with Sulfur Chemiluminescence Detection, Journal of Agricultural and Food Chemistry, 58 (2010) 9454-9462.

[3] E. Franco-Luesma, M.-P. Saenz-Navajas, D. Valentin, J. Ballester, H. Rodrigues, V. Ferreira, Study of the effect of H2S, MeSH and DMS on the sensory profile of wine model solutions by Rate-All-That-Apply (RATA), Food Research International, 87 (2016) 152-160.

[4] M.A. Segurel, A.J. Razungles, C. Riou, M. Salles, R.L. Baumes, Contribution of dimethyl sulfide to the aroma of Syrah and Grenache Noir wines and estimation of its potential in grapes of these varieties, Journal of Agricultural and Food Chemistry, 52 (2004) 7084-7093.

ACCEPTED MANUSCRIPT

[5] A. Escudero, E. Campo, L. Farina, J. Cacho, V. Ferreira, Analytical characterization of the aroma of five premium red wines. Insights into the role of odor families and the concept of fruitiness of wines, Journal of Agricultural and Food Chemistry, 55 (2007) 4501-4510.

[6] M. Ugliano, P.A. Henschke, Yeasts and wine flavour, Wine Chemistry and Biochemistry2009, pp. 313-392.

[7] D. Rauhut, Usage and formation of sulphur compounds, Biology of Microorganisms on Grapes, in Must and in Wine2017, pp. 255-291.

[8] D. Thomas, Y. Surdin-Kerjan, Metabolism of sulfur amino acids in Saccharomyces cerevisiae, Microbiology and Molecular Biology Reviews, 61 (1997) 503-532.

[9] P. Perpète, O. Duthoit, S. De Maeyer, L. Imray, A.I. Lawton, K.E. Stavropoulos, V.W. Gitonga, M.J. Hewlins, J. Richard Dickinson, Methionine catabolism in Saccharomyces cerevisiae, FEMS Yeast Research, 6 (2006) 48-56.

[10] O.A. Leppanen, J. Denslow, P.P. Ronkainen, Determination of thiolacetates and some other volatile sulfur-compounds in alcoholic beverages, Journal of Agricultural and Food Chemistry, 28 (1980) 359-362.

[11] N. Loscos, M. Segurel, L. Dagan, N. Sommerer, T. Marlin, R. Baumes, Identification of S-methylmethionine in Petit Manseng grapes as dimethyl sulphide precursor in wine, Analytica Chimica Acta, 621 (2008) 24-29.

[12] E. Vela, P. Hernandez-Orte, E. Franco-Luesma, V. Ferreira, Micro-oxygenation does not eliminate hydrogen sulfide and mercaptans from wine; it simply shifts redox and complex-related equilibria to reversible oxidized species and complexed forms, Food Chemistry, 243 (2018) 222-230.

[13] V. Ferreira, E. Franco-Luesma, E. Vela, R. López, P. Hernández-Orte, Elusive Chemistry of Hydrogen Sulfide and Mercaptans in Wine, Journal of Agricultural and Food Chemistry, 66 (2018) 2237-2246.

ACCEPTED MANUSCRIPT

[14] E. Franco-Luesma, V. Ferreira, Quantitative analysis of free and bonded forms of volatile sulfur compouds in wine. Basic methodologies and evidences showing the existence of reversible cation-complexed forms, Journal of Chromatography A, 1359 (2014) 8-15.

[15] G.Y. Kreitman, J.C. Danilewicz, D.W. Jeffery, R.J. Elias, Copper(II)-mediated Hydrogen Sulfide and thiol oxidation to disulfides and organic polysulfanes and their reductive cleavage in wine: mechanistic elucidation and potential applications, Journal of Agricultural and Food Chemistry, 65 (2017) 2564-2571.

[16] A.C. Clark, P. Grant-Preece, N. Cleghorn, G.R. Scollary, Copper(II) addition to white wines containing hydrogen sulfide: residual copper concentration and activity, Australian Journal of Grape and Wine Research, 21 (2015) 30-39.

[17] E. Vela, P. Hernández-Orte, E. Franco-Luesma, V. Ferreira, The effects of copper fining on the wine content in sulfur off-odors and on their evolution during accelerated anoxic storage, Food Chemistry, 231 (2017) 212-221.

[18] E. Franco-Luesma, V. Ferreira, Formation and Release of H2S, Methanethiol, and Dimethylsulfide during the Anoxic Storage of Wines at Room Temperature, Journal of Agricultural and Food Chemistry, 64 (2016) 6317-6326.

[19] S. Cheskis, E. Atar, A. Amirav, Pulsed-Flamed Photometer - A novel gas-chromatography detector, Analytical Chemistry, 65 (1993) 539-555.

[20] A. Amirav, H.W. Jing, Pulsed flame photometer detector for gas-chromatography, Analytical Chemistry, 67 (1995) 3305-3318.

[21] J.F. McGaughey, S.K. Gangwal, Comparison of 3 commercially available gas-chromatographic flame photometric detectors in the sulfur mode, Analytical Chemistry, 52 (1980) 2079-2083.

[22] X.W. Yan, Unique selective detectors for gas chromatography: Nitrogen and sulfur chemiluminescence detectors, Journal of Separation Science, 29 (2006) 1931-1945.

ACCEPTED MANUSCRIPT

[23] D. Rauhut, H. Kurbel, K. Macnamara, M. Grossmann, Headspace GC-SCd Monitoring of Low Volatile Sulfur-Compounds During Fermentation and in Wine, ANALUSIS, 26 (1998) 142-145.

[24] X.W. Yan, Detection by ozone-induced chemiluminescence in chromatography, Journal of Chromatography A, 842 (1999) 267-308.

[25] M.J. Navas, A.M. Jimenez, Chemiluminescent methods in petroleum products analysis, Critical Reviews in Analytical Chemistry, 30 (2000) 153-162.

[26] A. Technologies, https://www.agilent.com/en/products/gas-chromatography/selective-detectors/sulfur-chemiluminescence-detector, 2018.

[27] M. Mestres, O. Busto, J. Guasch, Chromatographic analysis of volatile sulphur compounds in wines using the static headspace technique with flame photometric detection, Journal of Chromatography A, 773 (1997) 261-269.

[28] J. Herszage, S.E. Ebeler, Analysis of Volatile Organic Sulfur Compounds in Wine Using Headspace Solid-Phase Microextraction Gas Chromatography with Sulfur Chemiluminescence Detection, American Journal of Enology and Viticulture, 62 (2011) 1-8.

[29] M. Mestres, M. Marti, O. Busto, J. Guasch, Simultaneous analysis of thiols, sulphides and disulphides in wine aroma by headspace solid-phase microextraction-gas chromatography, Journal of Chromatography A, 849 (1999) 293-297.

[30] X. Fan, C.H. Sommers, D.W. Thayer, S.J. Lehotay, Volatile sulfur compounds in irradiated precooked turkey breast analyzed with pulsed flame photometric detection, Journal of Agricultural and Food Chemistry, 50 (2002) 4257-4261.

[31] Y. Fang, M.C. Qian, Sensitive quantification of sulfur compounds in wine by headspace solid-phase microextraction technique, Journal of Chromatography A, 1080 (2005) 177-185.

[32] R. Lopez, A.C. Lapena, J. Cacho, V. Ferreira, Quantitative determination of wine highly volatile sulfur compounds by using automated headspace solid-phase microextraction and gas

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

chromatography-pulsed flame photometric detection - Critical study and optimization of a new procedure, Journal of Chromatography A, 1143 (2007) 8-15.

[33] S. Ullrich, S.K. Neef, H.G. Schmarr, Headspace solid-phase microextraction and gas chromatographic analysis of low-molecular-weight sulfur volatiles with pulsed flame photometric detection and quantification by a stable isotope dilution assay, Journal of Separation Science, 41 (2018) 899-909.

[34] R.A. Murray, Limitations to the use of solid-phase microextraction for quantitation of mixtures of volatile organic sulfur compounds, Analytical Chemistry, 73 (2001) 1646-1649.

[35] C. Haberhauer-Troyer, E. Rosenberg, M. Grasserbauer, Evaluation of solid-phase microextraction for sampling of volatile organic sulfur compounds in air for subsequent gas chromatographic analysis with atomic emission detection, Journal of Chromatography A, 848 (1999) 305-315.

[36] F. Lestremau, F.A.T. Andersson, V. Desauziers, Investigation of artefact formation during analysis of volatile sulphur compounds using Solid Phase Microextraction (SPME), Chromatographia, 59 (2004) 607-613.

[37] V. Carrascon, I. Ontanon, M. Bueno, V. Ferreira, Gas chromatography-mass spectrometry strategies for the accurate and sensitive speciation of sulfur dioxide in wine, Journal of Chromatography A, 1504 (2017) 27-34.

[38] J.M. Coelho, P.A. Howe, G.L. Sacks, A Headspace Gas Detection Tube Method to Measure SO_2 in Wine without Disrupting SO_2 Equilibria, American Journal of Enology and Viticulture, 66 (2015) 257-265.

[39] Y. Chen, J.A. Jastrzembski, G.L. Sacks, Copper-Complexed Hydrogen Sulfide in Wine: Measurement by Gas Detection Tubes and Comparison of Release Approaches, American Journal of Enology and Viticulture, 68 (2017) 91-99.

[40] M.Z. Bekker, M.P. Day, H. Holt, E. Wilkes, P.A. Smith, Effect of oxygen exposure during fermentation on volatile sulfur compounds in Shiraz wine and a comparison of strategies for

ACCEPTED MANUSCRIPT

remediation of reductive character, Australian Journal of Grape and Wine Research, 22 (2016) 24-35.

[41] N. Kontoudakis, A. Mierczynska-Vasilev, A. Guo, P.A. Smith, G.R. Scollary, E.N. Wilkes, A.C. Clark, Removal of sulfide-bound copper from white wine by membrane filtration, Australian Journal of Grape and Wine Research, 25 (2019) 53-61.

[42] M.Z. Bekker, G.Y. Kreitman, D.W. Jeffer, J.C. Danilewicz, Liberation of Hydrogen Sulfide from Dicysteinyl Polysulfanes in Model Wine, Journal of Agricultural and Food Chemistry, 66 (2018) 13483-13491.

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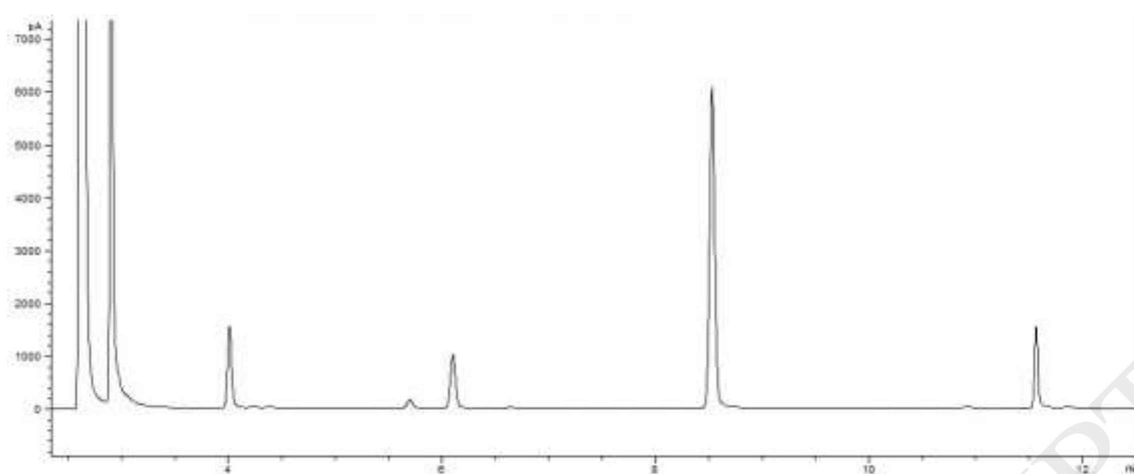

Figure 1a

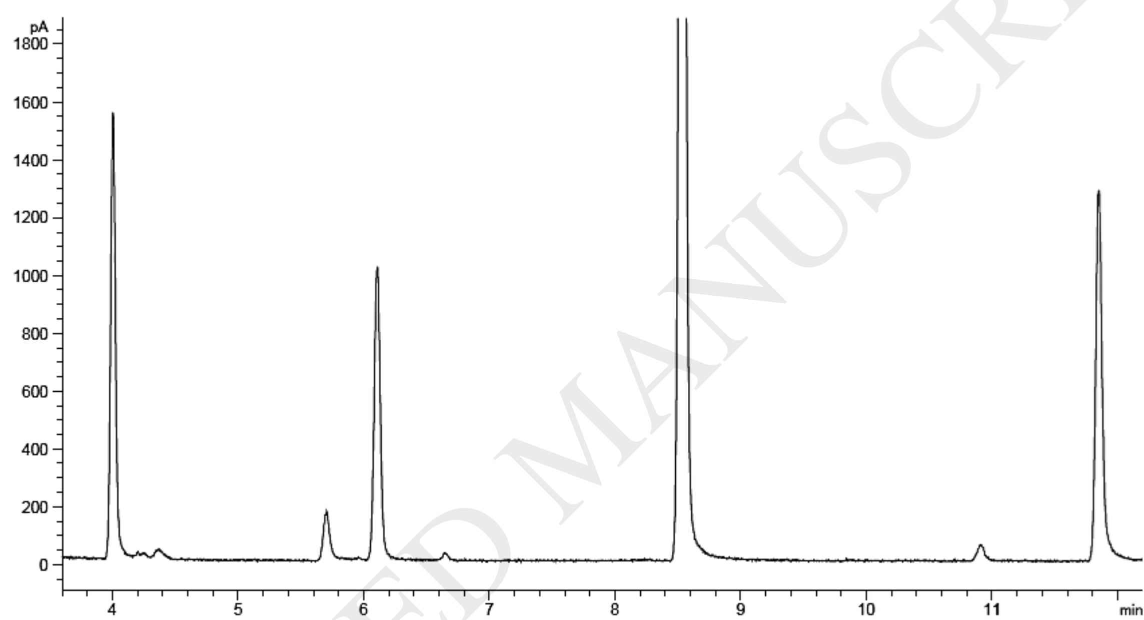

Figure1b.

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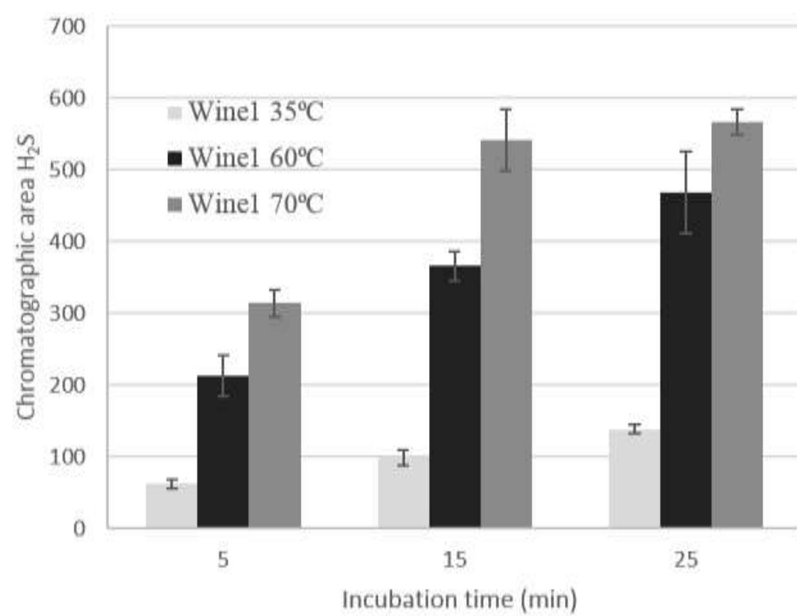

Figure 2a

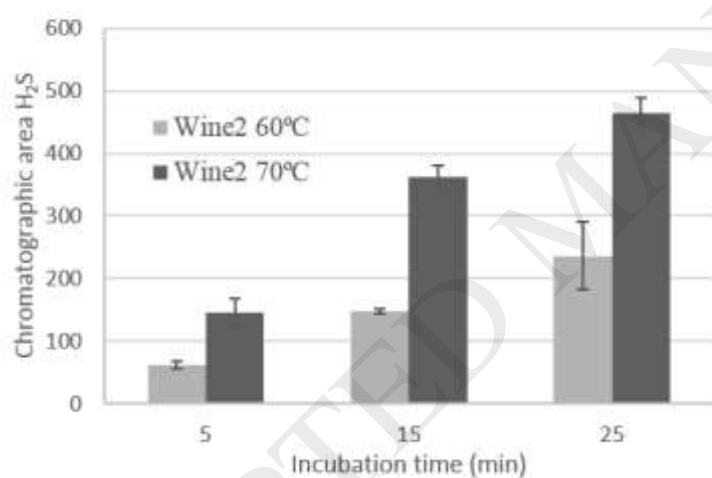

Figure 2b.

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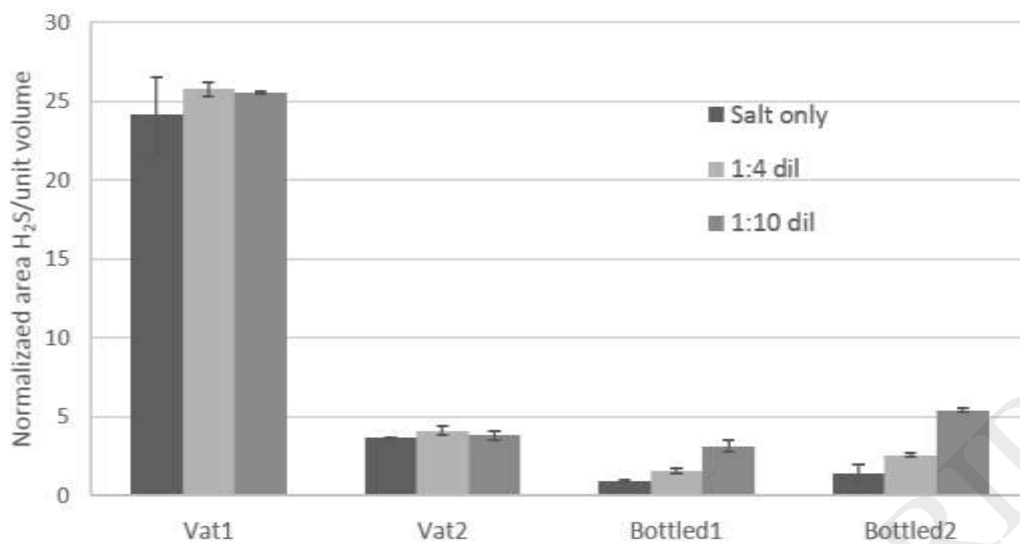

Figure 3

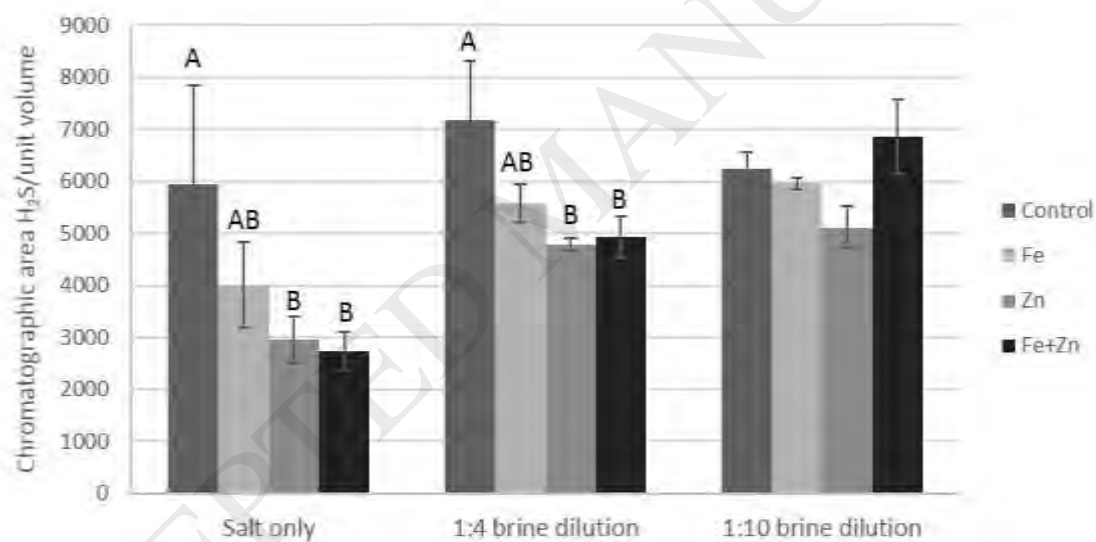

Figure 4

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Table 1: Optimal sampling-injection conditions for the three different procedures developed

	Free forms		Free and bound forms
	No cryofocusing	**With cryofocusing**	**BR**
Sample volume (mL)	12	12	1.2 (plus 10.8 mL Br)
Incubation Tª (°C)	30	30	70
Incubation time (min)	15	15	25
Agitation	5 s ON; 2 s OFF	5 s ON; 2 s OFF	5 s ON; 2 s OFF
Syringe Tª (°C)	40	40	80
Headspace volume (mL)	1	1	1
Injection volume (µL/s)	1000	30	1000
Split	1:20	1:2	1:15
Injector Tª (°C)	150	150	150

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Table 2: Comparison of method detection limits (µg/L) for free forms of VSCs and truly free SO_2 in the two procedures developed here and with the one working with a pFPD

Analyte	SCD No cryofocusing	SCD Cryofocusing	pFPD
H_2S	0.03	0.003	0.65
SO_2	51[*,a]	13[*,b]	-
MeSH	0.25	0.035	0.5
EtSH	0.37	0.06	0.5
DMS	0.84	0.07	1.0
DMDS	0.68	0.1	0.5
MeSAc	-	0.68	-
EtSAc	-	0.48	-
CS_2	-	0.01	-
DES	-	0.06	-
DEDS	-	0.05	-

*Free SO_2 at pH 3.4. [a]1.8 µg/L molecular SO_2. [b]0.46 µg/L molecular SO_2

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Table 3. Method precision. Ranges of RSD(%) obtained in the replicate analysis (n=3 or 4) of five different wines for the two procedures for free VSCs and truly free SO_2. Data of VSCs are in μg/L while for SO_2 are in mg/L

	No cryofocussing					With cryofocussing				
	QL	**low**	**RSD(%)**	**high**	**RSD(%)**	**QL**	**low**	**RSD(%)**	**high**	**RSD(%)**
H_2S	0.10	0.1-0.5	11-22	2.7-40	1.7-8.5	0.01	0.27-0.48	4.5-8.2	3.4-34	1.0-2.8
SO_2	0.17	0.2-0.5	3.2-5.9	1.4-7.3	1.5-2.9	0.05	0.52	10.9	1.0-5.0	1.3-5.9
MeSH	0.83	1.2-1.5	8.2-9.	2.1-4.8	2.6-3.1	0.12	1.3-1.9	0.2-4.9	2.57-4.16	0.8-2.5
EtSH	1.22			1.6	3.6	0.21	0.41	13.2	1.3-1.83	5.9-11.1
DMS	2.4	7.28	2.7	24-76	0.7-2.8	0.23	12.0-4.4	0.5-1.0		
DMDS	2.26	nd		nd		0.34	0.54	1.30		
CS_2	0.5	nd		nd		0.03	0.35-0.61	0.3-0.8		
MeSAc	20	nd		43	8.5	2.25			7.1-46.2	1.4-4.6
EtSAc	18	nd		nd		1.6	3.2-6.5	4.0-4.3		

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Table 4. Method linearity. Calibrated ranges and linearity data for the two developed procedures for the analysis of free VSCs and truly free SO_2. Calibration plots were built with model wines. Data of VSCs are in μg/L while for SO_2 are in mg/L

	No Cryo				**With Cryo**			
	Calibrated range	**r^2**	**Slope**	**Intercept**	**Calibrated range**	**r^2**	**Slope**	**Intercept**
H_2S	0.9-250	0.997	176.2	805	1-205	0.998	1721	2569
SO_2	0.5-200	0.995	318.3	-4.6	0.5-51	1	2705	12095
MeSH	0.85-25	0.994	50.84	16.5	0.5-15.4	0.993	455.7	-83.8
EtSH	1.2-24	0.997	54.72	-0.1	0.5-15.2	0.996	442.4	-28.7
DMS	2.5-246	0.999	30.25	13.4	0.9-198	0.998	303.6	-294
DMDS	1.7-19.6	0.999	40.17	10.6	0.4-14.4	0.998	248.6	37.5

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Table 5. Method figures of merit

	H_2S	MeSH	EtSH
Repeatablity ranges (RSD(%))	3.8-14% (6 samples)	(C<1.5 μg/L) 12.9-18.0% (2 samples) (C>1.5 μg/L) 5.8-8.7% (4 samples)	2.1% (1 sample)
Mean repeatability (RSD(%))	9.2% (6 samples)	(C<1.5 μg/L) 15.6% (2 samples) (C>1.5 μg/L) 7.5% (4 samples)	-
Detection limit (μg/L)	0.06	0.35	0.4
Quantitation limit (μg/L)	0.21	1.2	1.2
Calibrated range (μg/L)	1-200	1.2-13	1.3-15
r^2	0.997	0.994	0.995

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