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ARTICLE *in* ANALYTICA CHIMICA ACTA · APRIL 2014

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Development of a routine analysis of 4-mercapto-4-methylpentan-2-one in wine by stable isotope dilution assay and mass tandem spectrometry

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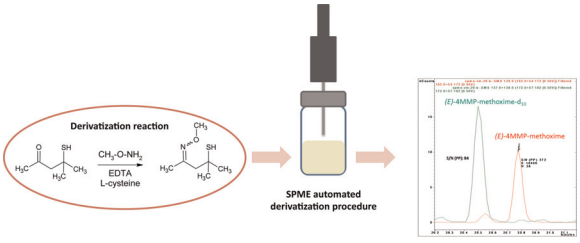
HIGHLIGHTS

- We proposed a routine method to analyze the 4-methyl-4-mercapto-pentan-2-one in wine.
- We developed the first method with sensitivity below the 4MMP perception threshold.
- We obtained an accurate method by using the stable isotope dilution assay approach.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 18 October 2013
Received in revised form 27 February 2014
Accepted 6 March 2014
Available online 11 March 2014

Keywords:
4-mercapto-4-methylpentan-2-one
Wine
Solid phase micro extraction
Stable isotope dilution assay
Varietal thiol
Validation

ABSTRACT

The 4-mercapto-4-methylpentan-2-one (4MMP) is a key aroma compound in wines, especially in Sauvignon Blanc ones. Its accurate quantification is quite difficult due to its traces levels and its reactivity in wine conferred by the thiol function. In this paper, we proposed a new method for its quantification in wine without any sample preparation, based on automated derivatization procedure by methoximation and SIDA–SPME–GC–MS/MS analysis. The derivatization procedure was adapted from a previously published method in order to decrease the amount of reagents and the volume of wine (only 3 mL are required). The use of SPME and the detection conditions have also been optimized to reach the best sensitivity as possible. The method was then validated according to the International Organization of Vine and Wine recommendations and exhibited excellent performances. Indeed, this method allowed us to quantify the 4MMP in wine at traces levels (LOD = 0.19 ng L^{-1}) with reproducible results (RSD < 15%) and a very good accuracy (recovery = 102%).

1. Introduction

The 4-mercapto-4-methylpentan-2-one (4MMP), first identified in Sauvignon Blanc [1] and Colombard [2] wines, is a potent odoriferous varietal thiol responsible for box-tree and blackcurrant bud notes in young white wines. Occurring at traces level, it has been later identified as a key odorant compound of white wines such as Scheurebe [3], Maccabeo [4], Gewurztraminer, Riesling, Muscat, Colombard, Petit Manseng and Tokay [5,6] and more recently in red wines [7]. The 4MMP exhibited a very low perception threshold equal to 0.8 ng L^{-1} in model medium [5]. Due to the presence of the thiol function, the 4MMP is highly reactive from a chemical point of view and requires the use of sophisticated quantification methods to obtain reliable results.

Few articles report the 4MMP quantification in wine [8,9] and most of them are particularly time-consuming, not sensitive enough and therefore, not usable as a routine method.

Abbreviations: 4MMP, 4-mercapto-4-methylpentan-2-one; SIDA, stable isotope dilution assay; SPME, solid phase micro-extraction; LOD, limit of detection; LOQ, limit of quantification; RSD, residual standard deviation.

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http://dx.doi.org/10.1016/j.aca.2014.03.004
0003-2670/

The main difficulty of the analysis lay in the 4MMP extraction from wine matrix and several methodologies has been proposed. The original method suggested by Tominaga et al. [10] was based upon a selective extraction of 4MMP from a wine dichloromethane extract using the reversible chelation of the thiol function by the sodium *p*-hydroxymercuribenzoate. Nevertheless, this method presents a major drawback concerning the 4MMP specificity since detection by GC–MS was performed only on one ion used both as quantifier and qualifier. Similar extraction procedures were later reported [11–13] and few of them had a limit of detection equal to the 4MMP perception threshold [12]. These methods were highly time consuming and not much safer for the analyst. Some improvements were later reported and consisted on derivatizing the 4MMP with pentaflurobenzyl bromide with [14,15] or without [16] a preliminary oximation step. Under these conditions, analysis sensitivity was increased since the reported limits of detection were below the 4MMP perception threshold. Recently, the ethyl propiolate was presented as a new derivatizing agent to quantify the 4MMP in wines [17]. Even if the sample preparation is much easier than those reported, the limit of detection equal to 24.5 ng L^{-1} was not sufficient to analyze the 4MMP in wines.

Concerning quantification procedures, few methods were based on stable isotope dilution assay (SIDA). Due to the non-commercial availability of labeled 4MMP, most of published methods used internal standards such as the 4-methoxy-2-methylbutan-2-thiol [10,13] or the 2-octanol [12]. Those standards are obviously not convenient to overcome the analyte losses during sample preparation since they do not have the same reactivity as4MMP. Thus, the best quantification procedure remains SIDA in term of accuracy.

Until now, there is no method allowing the quantification of 4MMP in wines with sensitivity below its perception threshold, an easy sample preparation and a quantification method base upon SIDA approach. For this purpose, we proposed for the first time a high throughput and fully validated method to quantify 4MMP in wine by SIDA–SPME–GC–MS/MS.

2. Materials and methods

2.1. Chemical and standards

The 4-mercapto-4-methylpentan-2-one (4MMP) was obtained from Sigma–Aldrich (Saint Quentin en Fallavier, France) and its labeled analogue (4MMP-d_{10}) has been synthesized according to the procedure reported by Kotseridis et al. [18]. Ethylenediaminetetraacetic acid (EDTA), L-Cysteine hydrochloride monohydrate and *o*-methylhydroxylamine hydrochloride were of analytical pure grade and were from Sigma–Aldrich (Saint Quentin en Fallavier, France). SPME fiber (Divinylbenzene/Carboxen/Polydimethylsiloxane) (DVB/CAR/PDMS) was obtained from Supelco.

2.2. Model wine preparation

Model wine was composed of water (800 mL), ethanol (120 mL) and tartaric acid (3,5 g L^{-1}). The pH of both was adjusted to 3.5 using potassium carbonate.

2.3. Analysis of 4MMP

2.3.1. Derivatization procedure

To an aliquot of wine (3 mL), we added successively deionized water (7 mL), the internal standard (4MMP-d_{10}) at 52.3 ng L^{-1} then sodium chloride (4 g). Then, derivatizing agents were successively added to the SPME vial as follow: EDTA (25 mg), L-cystein hydrochloride monohydrate (16 mg) and *o*-methylhydroxylamine hydrochloride (10 mg). The derivatization reaction was then carried out using an automatic CombiPal system (CTC Analytics, Zwingen, Switzerland). In practice, vials were stirred for 45 min at 55 °C, then SPME extraction on a DVB/CAR/PDMS fiber, previously conditioned at 250 °C for 12 min, was conducted for 30 min at 55 °C. Finally, the compounds were desorbed into the GC inlet at 250 °C for 3 min.

2.3.2. GC–MS/MS conditions

Analyses were performed using a GC–MS/MS system that consisted of a GC 3800 (Varian) coupled with an ion trap MS4000 (Varian). Analytes were separated on a DB WAX capillary column (60 m × 0.25 mm × 0.25 μm) from Supelco. The oven temperature program was as follow: 35 °C for 3 min, increased to 80 °C at 15 °C min^{-1}, increased to 130 °C at 3 °C min^{-1} then increased to 245 °C at 20 °C min^{-1} for 10 min. The carrier gas was helium with a constant flow rate of 1 mL min^{-1} and the injector temperature was set at 250 °C. Injection was performed in splitless mode for 3 min then a split of 1/20 was operated. The trap and transfer line temperatures were set at 150 °C and 170 °C respectively. Ionization was performed in positive chemical ionization using isobutane (pressure 1.5 bar). Detection was performed in MS/MS conditions in resonant mode. The *m/z* 163 and *m/z* 173 ions were chosen for 4MMP and 4MMP-d_{10} as parent ions respectively with an isolation window of 3 amu. The excitation amplitude was set at 0.6 V and the excitation storage level was adjusted to *m/z* 53.5 ($q = 0.3$) and *m/z* 56.8 ($q = 0.3$) for *m/z* 163 and *m/z* 173 respectively. The scan ranged from *m/z* 57 to *m/z* 182. The quantification was performed using *m/z* 128 and 138 as daughter ions for the natural and labeled compounds respectively.

2.4. Validation procedure

The analytical method validation was performed by evaluating the following parameters: matrix effect, linearity, accuracy, repeatability, intermediate reproducibility, limits of detection (LOD) and quantification (LOQ).

Matrix effect was measured by comparing the signal obtained in a model and real samples (red, white and rosé wines) spiked with 4MMP at the same concentration. Real samples were spiked with 4MMP at 0 and 20 ng L^{-1} in triplicate and the signal corresponding to the enrichment was compared with those obtained for a model wine spiked at 20 ng L^{-1}.

Linearity was calculated by spiking model wine with 4MMP at different levels and by plotting the 4MMP to 4MMP-d_{10} ratios against the corresponding area ratios. Linearity was evaluated by a lack of fit test. For this purpose, 9 samples (model wine spiked with 4MMP at 9 levels of concentrations (0–100 ng L^{-1}) and always 52.3 ng L^{-1} of 4MMP-d_{10}) were analyzed in triplicate.

Accuracy, repeatability and intermediate reproducibility were measured by spiking real samples with 4MMP at 5 levels and results were expressed as the recovery (concentrations calculated from calibration compared with theoritical concentrations, expressed in %) and mean RSD (%) respectively. In practice, real samples spiked with 4MMP at 5 levels (10, 20, 46, 70 and 100 ng L^{-1}) were analyzed in triplicate and under repeatability conditions. For intermediate reproducibility, spiked samples with 4MMP at 20 ng L^{-1} were prepared and analyzed in triplicate on 3 different days and by 2 different operators.

LODs and LOQs were evaluated as the lowest concentration at which a signal-to-noise (S/N) ratio greater than 3 and 10, respectively, could be consistently measured in spiked wine ($n = 3$) samples.

3. Results and discussion

3.1. Characterization of 4MMP derivatives

All the published methods, that have sensitivity close to the 4MMP perception thresholds, are based upon a derivatization procedure. Indeed, the detection of 4MMP by mass spectrometry is difficult due to its important fragmentation, i.e. the weak abundance of specific high mass fragments in its mass spectrum, either in electron impact ionization or chemical ionization. So, the use of derivatization increased the molecular mass of 4MMP and so its detection by MS/MS.

Two types of derivatization procedures have been reported. The first one involved the derivatization of the thiol function though anucleophilic attack on either pentafluorobenzyl bromide (PFBBr) [16] or on ethyl propiolate [17]. Whatever the kind of derivatization reagents used, the authors reported that the 4MMP reacted at very slow rates probably due to the intra-molecular hydrogen bond between the thiol and the carbonyl functions. The second strategy of derivatization consisted of taking advantage of this intra molecular bond by forming a nucleophilic attack of the *o*-methylhydroxylamine on the 4MMP carbonyl function [14].

We decided to take advantage of the Mateo-Vivaracho et al. [14] work by adjusting the methoximation procedures. We used a CTC Combipal to make the derivatization procedure easier for operators. The derivatization reaction led to the formation of two 4MMP-methoxime derivatives that corresponds to the (*E*) and (*Z*) isomers (Fig. 1). Due to the steric hindrance, the isomer (*E*) was preferentially formed during the reaction and corresponded to about 80% of the sum of both isomers as already reported [14]. Consequently, quantification was performed only on the (*E*) isomer signal for both natural and labeled 4MMP-methoxime.

3.2. MS/MS conditions optimization

In literature, the 4MMP was typically detected by mass spectrometry except for one study where detection was carried out by an atomic emission detector [11]. To reach the best sensitivity possible, it is important to study the influence of ionization on the 4MMP-methoxime. Previous work used negative chemical ionization (NCI) with methane in combination with SIM mode [7,14–16,19] to detect the 4MMP-methoxime derivatives. In regard to those results, we decided to study the influence of ionization modes in order to develop MS/MS conditions.

We characterized the natural and labeled 4MMP-methoxime by GC–MS in both electron impact (EI) ionization and positive chemical ionization (PCI) using isobutane. As shown in Fig. 2, the mass spectrum of 4MMP-methoxime acquired under EI conditions showed a lot of fragmentations and no ion in terms of specificity and intensity that can be used for further MS/MS experiments. On the contrary, the same analysis performed in PCI conditions allowed the identification specific and intense ions: the *m/z* 162 signal corresponded to the $[M+H]^+$ ion and the *m/z* 128 signal referred to the neutral loss of H_2S. The *m/z* 162 represented very good candidate to perform further MS/MS analysis on 4MMP-methoxime. So the MS/MS re-fragmentation of the ion *m/z* 162 of

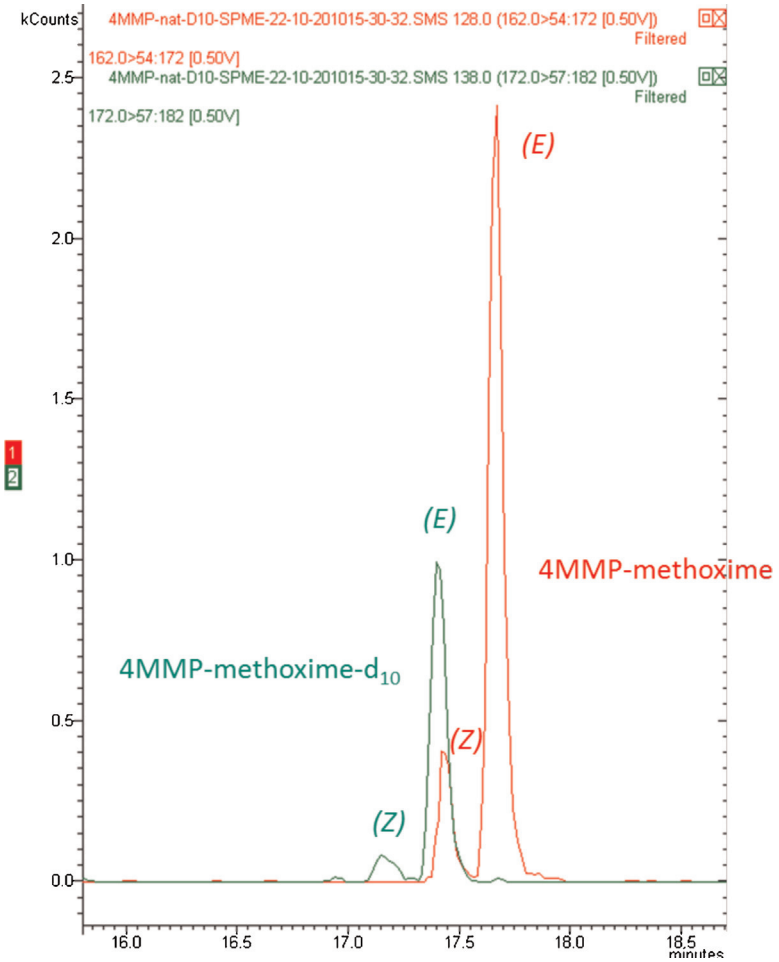

Fig. 1. Extract Ion Chromatogram of *m/z* 163 → *m/z* 128 and *m/z* 173 → *m/z* 138 for natural and labeled 4MMP-methoxime respectively.

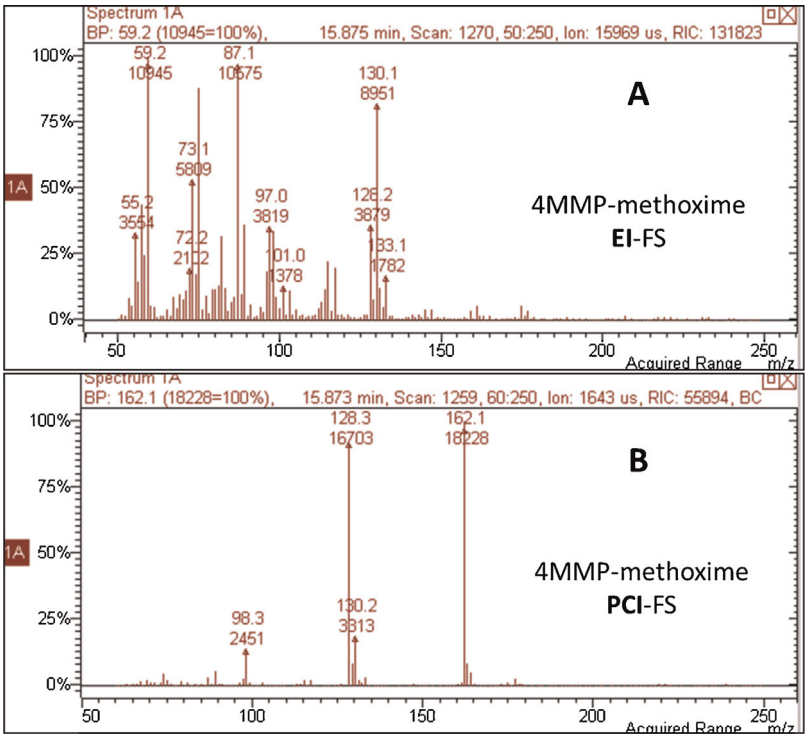

Fig. 2. Comparison of 4MMP-methoxime mass spectra acquired in EI (A) and PCI (B) mode. (FS: full scan).

4MMP (172 for deuterated 4MMP) provides us the daughter ions *m/z* 128 (138), *m/z* 98 (108), *m/z* 163 (173). We choose the ion *m/z* 128 (138) as quantifier and the two others as qualifiers.

In a second time, MS/MS conditions have been optimized using *m/z* 162 and *m/z* 172 ions as parent ions for natural and labeled 4MMP-methoxime respectively. Optimization was carried out by varying the waveform type (resonant or non-resonant), the excitation storage level and the excitation amplitude through the automatic method development process proposed by the Varian workstation software. The best MS/MS conditions allowed us to quantify 4MMP-methoxime in a complex matrix (Fig. 3) due to a very specific and intense transition (*m/z* 162 → *m/z* 128 and *m/z* 172 → *m/z* 138 for natural and labeled 4MMP-methoximes). The identification was performed using the specific 2nd order mass spectrum pattern (Fig. 4). We observed as a result of this re-fragmentation process the formation, the disappearance of *m/z* 162 (172) and the formation of daughter ion *m/z* 163 (173). The detection by MS/MS and the ionization in PCI mode we developed increased the sensitivity of 4MMP analysis (LOD close to 0.2 ng L^{-1}) in comparison with others published methods.

3.3. Validation procedure

The analytical validation was performed according to the recommendations of the International Organization of Vine and Wine (OIV) [20]. The results were presented in the following sections.

3.3.1. Matrix effect

Matrix effects have been calculated by comparing the 4MMP-methoxime signal in model and real samples that have been fortified with 4MMP at 20 ng L^{-1}. The calculation of the *Z*-score parameter allowed determining if the analysis was specific or not. If the *Z*-score is higher than 2, the method is considered as not specific and matrix effect is not negligible.

As shown in Table 1, no matrix effect has been observed between model wines and real fortified samples with synthetic 4MMP. Indeed, *Z*-scores values were systematically below 1 that demonstrated the specificity of our detection conditions. At this stage of the study, the absence of matrix effect enabled calibration in model conditions and thus made the analysis procedure easier for operators.

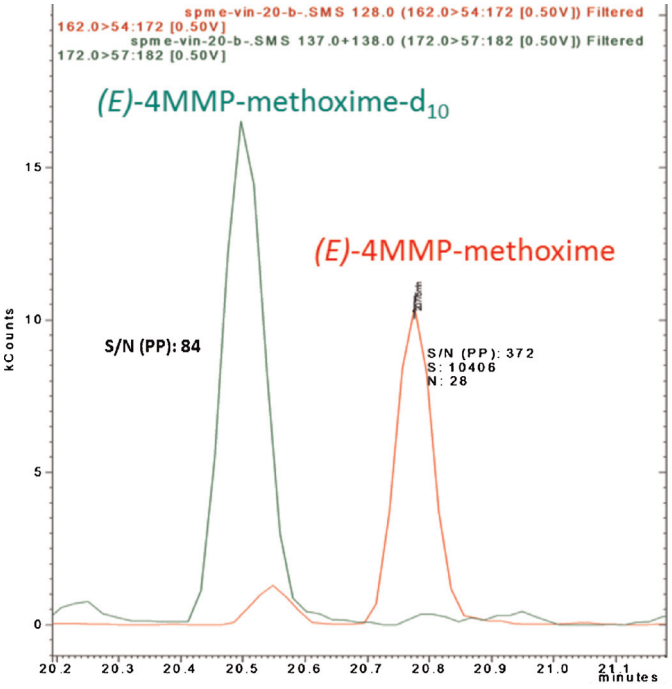

Fig. 3. Chromatogram of a white wine spiked with 4MMP at 20 ng L^{-1} and 4MMP-d_{10} at 50 ng L^{-1} (S/N referred to the signal to noise ratio using the "peak to peak" approach).

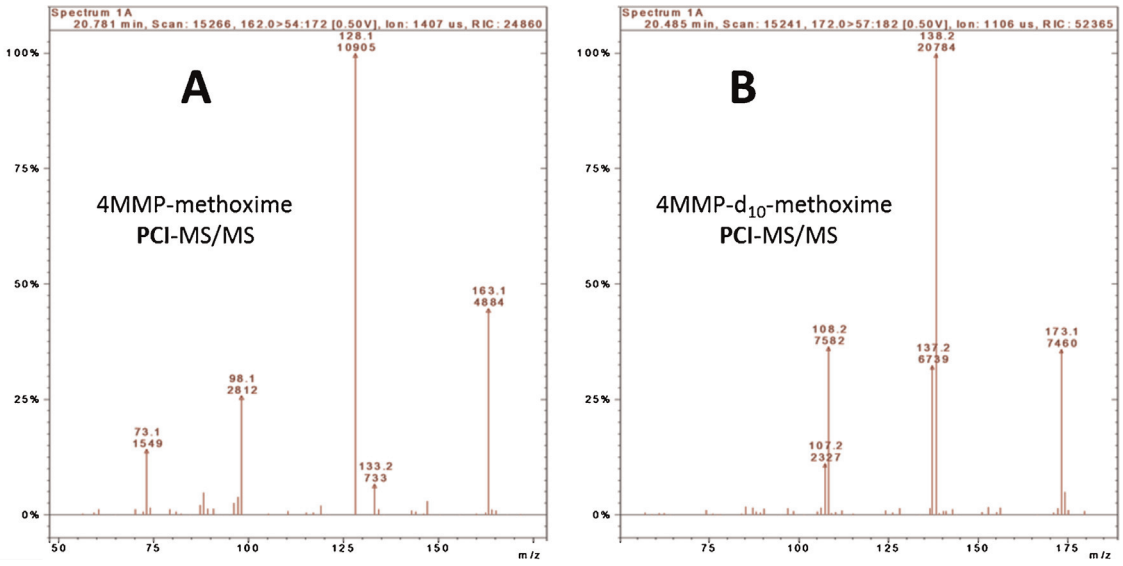

Fig. 4. Second order mass spectra of natural (A) and labeled (B) 4MMP-methoximes using PCI ionization mode.

3.3.2. Linearity

Linearity study was conducted in spiked model wine with synthetic 4MMP at levels ranging from 0 to 100 ng L^{-1} (nine calibrators) and analyzed in triplicate. ISTD have been introduced at 50 ng L^{-1} that is to say in the middle range of the calibration. To define the regression model, we performed a lack of fit test on each data series by plotting the concentration ratios against the corresponding area ratios (Table 1). Over the considered range of concentration, calibration curves were linear. The slope was close to 1 due to the use of SIDA approach. Indeed, the labeled internal standard and the natural analyte exhibited a similar response factor involving a slope close to 1.

3.3.3. Accuracy

Accuracy was evaluated by adding known amounts of 4MMP into three different wines (white, rosé and red varieties) at different concentration levels (10, 20, 46, 70 and 100 ng L^{-1}). In parallel, control samples (no spiked wines) were analyzed to distinguish the natural amount of 4MMP from the added one. Recoveries for the three types of matrices were satisfactory as the averaged value range from 99 to 102% according to the level of concentration. Those very satisfactory results showed that the use of SIDA could help to overcome the loss of analyte during the sample preparation.

Table 1
Analytical performances.

	Wines		
	White	Rosé	Red
Matrix effect (*Z*-score)	0.610	0.977	0.315
Regression model: lack of fit test	Linear		
Equation (range: 1 nM–40 nM	$Y = 0.9648X + 0.0141$		
R^2	0.9979		
Mean accuracy[a] (recovery %)			
20ng L^{-1}	99		
70ng L^{-1}	102		
Mean repeatability[a] (RSD %)	7		
Mean intermediate reproducibility[a] (RSD %)	15		
Mean LOD[a] (ng L^{-1})	0.19		
Mean LOQ[a] (ng L^{-1})	0.64		

[a] Values are averaged for the three different matrices except for the matrix effect evaluation.

3.3.4. Precision

Precision referred to the measurement of repeatability and reproducibility and was expressed by residual standard deviation values (RSD) under repeatability and reproducibility conditions. In our case, we only evaluated the intermediate reproducibility for obvious technical constraints by varying the analysis dates and the operators. In practice, precision was estimated at 5 levels of concentrations and results were shown in Table 1. These results showed that the SIDA method we developed exhibited excellent repeatability and reproducibility with RSD below to 15% for both parameters.

3.3.5. LOD and LOQ

Limits of detection and quantification were measured by considering that signal to noise ratio should be equal to 3 and 10 respectively. Values were presented in Table 1 and showed the high sensitivity of our method. Indeed, LOD and LOQ were below the 4MMP perception threshold of 0.8 ng L^{-1}.

3.3.6. Analysis of some wines

This methodology has been applied to the analysis of 18 young Sauvignon Blanc wines (one replicate) from Loire valley (Fig. 5).

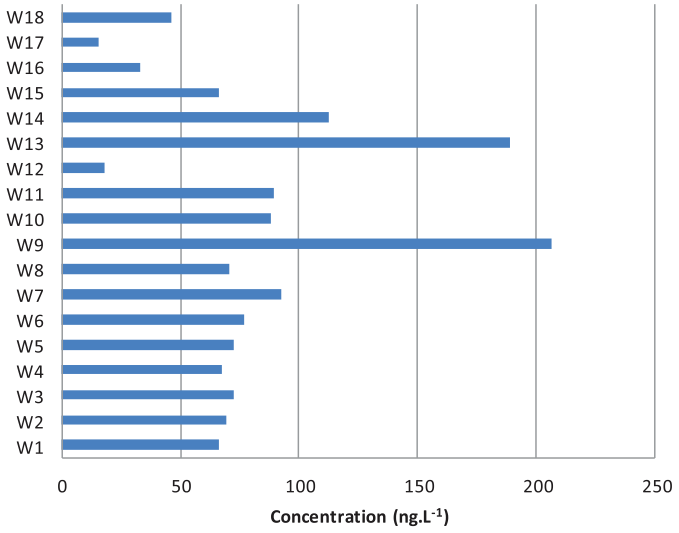

Fig. 5. 4MMP concentrations found in 18 Sauvignon Blanc wines from Loire valley.

Average 4MMP concentration was close to 80 ng L^{-1} which is consistent with previous work [6]. Three wines exhibited particularly high 4MMP content up to 200 ng L^{-1} giving important fruity notes to these wines.

4. Conclusion

This is the first method enabling high throughput analysis of 4MMP in wine using a very small volume of sample and exhibiting a very moderate cost. This routine analysis exhibited very impressive accuracy and precision due to the use of SIDA procedure and can be easily reproduced by an inexperienced operator. In addition, this is the only published and validated method permitting the quantification of 4MMP in wines at levels below its perception threshold.

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