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Validation of a nanoliquid chromatography–tandem mass spectrometry method for the identification and the accurate quantification by isotopic dilution of glutathionylated and cysteinylated precursors of 3-mercaptohexan-1-ol and 4-mercapto-4-methylpentan-2-one in white grape juices

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ABSTRACT

A rapid nanoLC–MS/MS method was developed and validated for the simultaneous determination of glutathionylated and cysteinylated precursors of 3-mercapto-hexan-1-ol (3MH) and 4-methyl-4-mercaptopentan-2-one in grape juice using stable isotope dilution assay (SIDA). The analytes were extracted from must using a cation exchange resin and purified on C18 cartridges. They were chromatographically separated on a reverse phase column and finally analyzed by tandem mass spectrometry in selected reaction monitoring mode (SRM) using deuterated analogues as standards except for glutathionylated conjugate of 4MMP which was analyzed by external calibration. The method was validated according to the International Conference on Harmonization recommendations by determining linearity, accuracy, precision, recovery, matrix effect, repeatability, intermediate reproducibility, LODs and LOQs.

Calibration for each precursor was determined by performing Lack-of-Fit test and the best fitting for 3MH precursors was a quadratic model whereas a linear model was better adapted for 4MMP precursors. All calibration curves showed quite satisfactory correlation coefficients ($R^2 > 0.995$ for SIDA quantification and $R^2 > 0.985$ for external calibration). Quantification by SIDA and external calibration allowed a high level of accuracy since the averaged value ranged from 80 to 108%. Quantification of aroma precursors was accurate and reproducible over five days since intermediate precision (same analyst, same sample and same apparatus), which was evaluated by the calculation of RSD was inferior to 16%. Limits of quantification for G3MH and G4MMP were closed to 0.50 and 0.07 nmol/L and as 4.75 and 1.90 nmol/L for Cys3MH and Cys4MMP respectively.

This method was applied to the quantification of precursors into several types of grape juices: Melon B., Sauvignon, Riesling and Gewurztraminer.

1. Introduction

Three volatile thiols are known to be ones of the major positive contributors of wine aroma: 4-methyl-4-mercapto-pentan-2-one (4MMP) [1], reminiscent of box tree and blackcurrant bud, 3-mercapto-hexan-1-ol (3MH) and its acetate (3MHA)[2], that are responsible of the fruity and citrus notes of lots of wine. These thiols are formed from odorless precursors naturally occurring in must and grapes.

The well-known and accepted biogenesis pathway of 3MH and 4MMP involves the cleavage of the corresponding cysteinylated conjugates (Cys4MMP and Cys3MH) present in grapes, by the carbon-sulfur lyase activity of yeast during alcoholic fermentation

Abbreviations: SIDA, stable isotope dilution assay; G3MH, S-3-(hexan-1-ol)-glutathione; G4MMP, S-3-(4-methyl-4-mercaptopentan-2-one)-glutathione; Cys3MH, S-3-(hexan-1-ol)-cysteine; Cys4MMP, S-3-(4-methyl-4-mercaptopentan-2-one)-cysteine; SRM, selected reaction monitoring; LSD, least significant difference test; CID, collision induced dissociation; BSTFA, N,O-bis-(trimethylsilyl)-trifluoroacetamide; TFA, trifluoroacetic acid.

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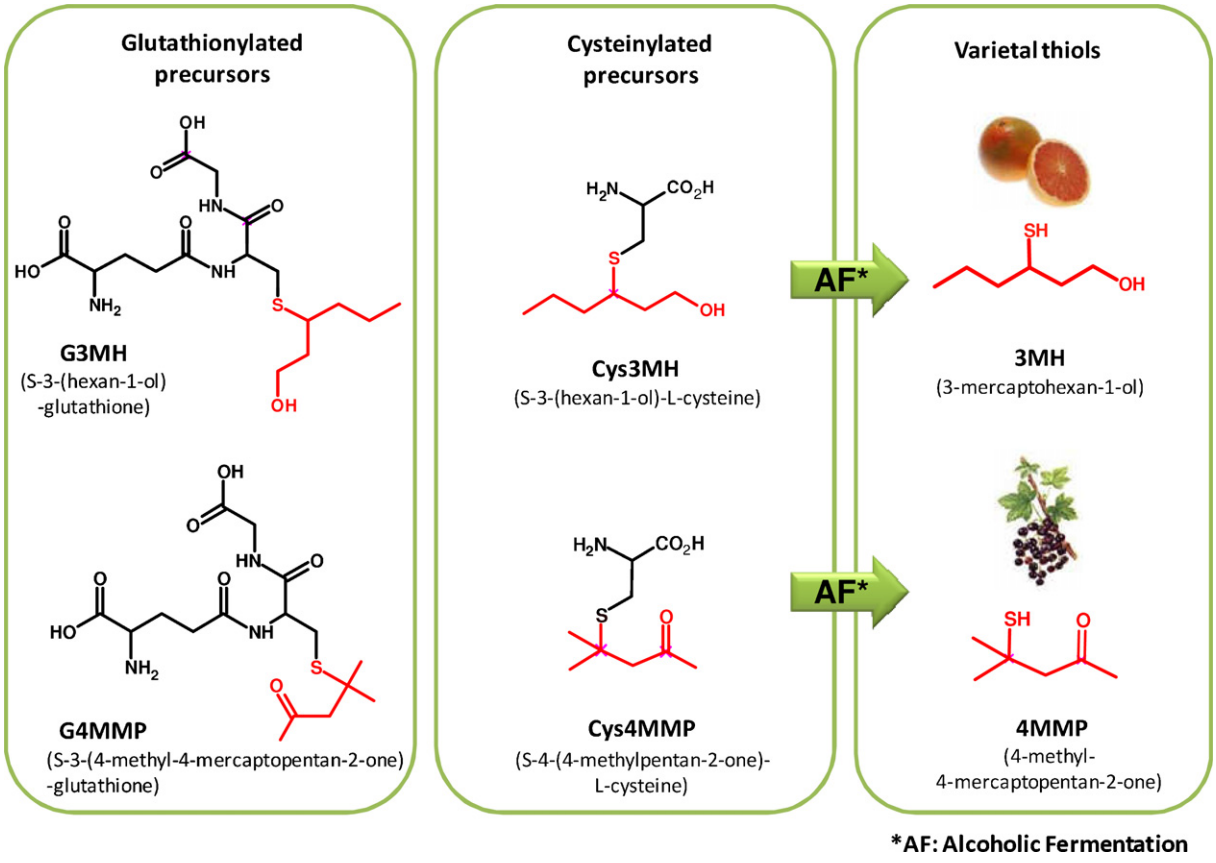

Fig. 1. Glutathionylated and cysteinylated precursor of 3MH and 4MMP, varietal thiols reminiscent of fruity notes and released during alcoholic fermentation.

[3,4] (Fig. 1). The conversion yield is generally below 1% and can only correspond in certain cases to 3–7% of the total 3MH produced [5].

Recently, other biogenesis pathways were proposed. Firstly, an alternative pathway was demonstrated from *trans*-2-hexen-1-al and mesityl oxide leading to the corresponding thiols by sulfur addition during fermentation [6]. However, the mechanism of that conversion has not been yet elucidated. Another possible biogenesis pathway of 3MH would be the degradation of glutathionylated pro-precursor into cysteinylated precursors in grape juice by α-glutamyltranspeptidase [7]. Glutathionylated conjugates of 4MMP and 3MH were formally identified in musts of different varieties [8,9], and the direct conversion of the S-3-(hexan-1ol)-glutathione (G3MH) by yeast during fermentation was also demonstrated in Sauvignon Blanc grape juice [9] (Fig. 1).

Further studies are necessary to better elucidate the biogenesis pathway that involves the gluthathionylated conjugates and the proportions of these different pathways. Such studies will require the use of an accurate and sensitive analytical method allowing the detection and the quantification of the different precursors in must at traces levels. The quantification of *trans*-2-hexen-1-al, a volatile compound present in must at mg/L level, is quite easy and well documented [10,11]. However, the direct and accurate quantification of cysteinylated and glutathionylated conjugates is more problematical.

Several analytical methods were reported for the identification and quantification of cysteinylated precursors in must. GC–MS methods were described and required either a cleavage step [12,13], or a derivatization step using N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) [13] or ethylchloroformate [14]. These methods are time-consuming and require practical skills. More recently, Cys3MH has been quantified in Petite Arvine must using HPLC-MS [15]. But to our knowledge, there is no published method allowing the simultaneous quantification of both glutathionylated and cysteinylated precursors in grape juice by stable isotope dilution assay.

Most of the methods previously described, were based upon gas-chromatography analysis which is widely used in food and wine industry for volatile compounds whereas for such non-volatile compounds, liquid chromatography represents the most appropriate technique for quantification at μg/L levels. However, to quantify thiols precursors present in must at ng/L levels (especially for Cys4MMP [12]) with a sufficient sensitivity, nanoliquid chromatography, firstly reported by Karlsson and Novotny in 1988 [16], seemed to be the most convenient method. It was already used for the quantification of biogenic amines in wines [17], pesticides in baby foods [18] and glycyrrhizin and glycerrhetic acid in licorice roots and candies [19].

Thus, the aim of this work was to develop and validate a rapid nanoLC–MS/MS method that would allow the accurate determination of precursors in must from different grape varieties.

2. Experimental procedures

2.1. Generality

For synthesis experiments: All solvents were analytical pure grade (>98%). Reduced glutathione was purchased from Duchefa Biochemie (Amsterdam, The Netherlands). Trifluoroacetic acid, di-*tert*-butyldicarbonate, dimethylformamide dineopentylacetal, *trans*-2-hexenal, sodium borodeuteride or borohydride, manganese dioxide, hexyn-1-ol, deuterium and dithiothreitol were purchased from Sigma Aldrich (St Quentin Fallavier, France). Palmitoyl chloride, ethyl acetate, *tert*butanol, triethylamine,

Table 1
Purities of synthetic standards determined by 1H NMR and calibration points made by diluting stock solutions in water.

Concentrations (nmol/L) (Purities (%))	Compounds			
	G3MH–G3MHd_2 (16–6)	G4MMP (26)	Cys3MH–Cys3MH d_2 (8–29)	Cys4MMP–Cys4MMPd_6 (9–48)
Standard 1	0.1–5	0.1	1–50	1–50
Standard 2	0.3–5	0.3	3–50	3–50
Standard 3	0.6–5	0.6	6–50	6–50
Standard 4	0.8–5	0.8	8–50	8–50
Standard 5	1–5	1	10–50	10–50
Standard 6	2–5	2	20–50	20–50
Standard 7	4–5	4	40–50	40–50
Standard 8	6–5	6	60–50	60–50
Standard 9	8–5	8	80–50	80–50
Standard 10	10–5	10	100–50	100–50

N-Boc-cysteine and triethylsilane were obtained from Fluka (St. Quentin Fallavier, France), tetrahydrofurane, dichloromethane, hexane and pentane from Riedel de Haen (St. Quentin Fallavier, France), acetic acid, toluene, dioxane and sodium sulfate from Merck (Darmstadt, Germany). All gases, nitrogen, helium were purchased from Air Product (Paris, France). Flash chromatographies were performed using Merck (Darmstadt, Germany) silica gel (grade 9385, 230–400 Mesh).

For extraction/purification experiments and LC analyses: Cation exchange resin DOWEX 50WX4-100, and hydrochloric acid were purchased from Sigma (St. Quentin en Fallavier, France). Ammonium dihydrogenphosphate was purchased from Acros Organics (Halluin, France). Cartridges C18 Sep-Pak were purchased from Waters (Baden, Switzerland). Acetonitrile was purchased from Biosolve (Valkenswaard, The Netherlands). Formic acid was purchased from Fluka (Epalinges, Switzerland) and methanol from Merck (Darmstadt, Germany).

2.2. Synthesis of natural and labeled standards

S-3-(hexan-1-ol)-glutathione (G3MH/G3MH d_2) [9,20], S-3-(4-methyl-4-mercaptopentan-2-one)-glutathione (G4MMP)[8], S-3-(hexan-1-ol)-cysteine (Cys3MH/Cys3MH d_2)[14] and S-3-(4-methyl-4-mercaptopentan-2-one)-cysteine (Cys4MMP/Cys4MMP d_6)[14] were synthesized according to the respective described methods. Synthetic natural and deuterated compounds were characterized and quantified by ^{1}H NMR using trimethylsilylpropionic acid as internal standard. Purities (Table 1) ranged from 6 to 48% according to the percentage of TFA (trifluoroacetic acid) salt occurring in each compound. The presence of TFA salt did not interfere with the analysis of thiol precursors since it was removed during the desalting step on reverse phase C18 cartridge.

2.3. Samples

Two types of samples were used for this study: grape juices prepared in our labs from fresh grapes, and industrial musts collected in several wineries.

Grape juice samples were prepared by crushing fresh berries of Melon B. and Sauvignon, under vacuum, in presence of benzene sulfinic acid (1 mg/mL) and sodium metabisulfite (4.5 mg/mL) as described by Cheynier et al. [21]. The grape samples were collected in different vineyard of the Loire Valley during the harvest in 2007 and 2008. After crushing, the stabilized grape juice obtained was stored at −20 °C until analysis.

Industrial musts were collected after the crushing step in different wineries from the Loire Valley for Sauvignon and in Alsace for Riesling and Gewurztraminer. After being collected, musts were stored at −20 °C without any further stabilization until analysis.

2.4. Sample preparation

Extraction was performed on an aliquot of must or grape juices (1200 μL) using DOWEX 50WX4-100 ion exchange resin (50 mg) in a micro-column. Resin was first conditioned by water (600 μL, 15 min), then HCl 2 M (600 μL, 15 min) and finally washed using water (4 mL).

Centrifugated samples (1200 μL) were spiked with internal standards: G3MH d_2 at 5 nmol/L and Cys3MH d_2 and Cys4MMP d_6 at 50 nmol/L, in initial volume of must. Then they were loaded on the resin and washed with water (1 mL). Elution was performed using an ammonium buffer ($NH_4^+H_2PO_4^-$, 1 M, 1 mL) and extracts were then purified on C18 cartridge (Sep-Pak) previously conditioned with MeOH (1 mL) then water (2 × 1 mL). Extracts were loaded on the cartridges, washed with water (600 μL) and the final elution was performed using MeOH (600 μL). The final extracts were concentrated to dryness, and then dissolved into an accurate volume of water (50 μL).

2.5. NanoLC–MS/MS analyses

Nanoliquid chromatography was performed on a Waters Acquity system coupled to a Thermo TSQ Vantage EMR (Extended Mass Range) mass spectrometer.

Analytes were first trapped (3 min at 5 μL/min) on a NanoEase Atlantis dC18 pre-column (Waters, 0.18 mm × 23.5 mm, 5 μm) and then separated on a Magic-C18 column (75 μm × 100 mm, 5 μm). The flow analytical flow rate was maintained at 800 nL/min. Mobile phases consisted of (A) water with 0.1% of formic acid and (B) acetonitrile with 0.1% of formic acid. The gradient profile started from 0.5% B for 1.5 min, increased to 5.0% B in 0.5 min, increased to 30.0% B in 16 min, increased to 40% B in 2 min and then increased to 85.0% B in 6 min for 2 min before returning to initial conditions.

The mass spectrometer was a triple-quadrupole, with electrospray ion source operated in positive mode. The spray voltage was maintained to 1.0 kV and the source temperature was 200 °C. Ionization and fragmentation parameters were optimized by infusing reference compounds at 2500 nmol/L in solution with MeOH (50%) and formic acid (0.1%). At such concentration, ionization of analytes induced a stable and intense spray allowing the optimization of key parameters as parents masses, S-lens and collision energy values. Fragmentations were studied and mass spectrometers parameters were optimized as shown in Table 2.

2.6. Calibration

Stock calibrant solutions were prepared by dissolving in water the synthesized compounds precisely weighed on a precision balance (Mettler Toledo, AX26 Comparator). Two stock solutions were prepared: one containing the 4 natural precursors, and the other

Table 2
MS/MS parameters (Quantifier ion in bold (*relative intensities*)) obtained by infusing solution of standards at 2500 nmol/L (mixture composed by 50% MeOH, 50% Water and 0.1% Formic acid) into mass spectrometer in manual tune mode (triple quadrupole, Thermo TSQ Vantage).

Parent ions (Da)	Daughter ions (Da)	Collision energy (V)	S-lens (V)	Scan segment (min)	Compounds
220.121	98.99 *(30.83)*	5	65		
220.121	105.05 *(1.38)*	5	65	5–9.5	Cys4MMP
220.121	**122.20** *(100)*	6	65		
226.161	105.09 *(100)*	11	66		
226.161	122.03 *(61.87)*	5	66	5–9.5	Cys4MMPd_6
226.161	168.20 *(1.51)*	12	66		
222.127	83.19 *(64.30)*	12	67		
222.127	101.12 *(10.87)*	8	67	6.5–11.5	Cys3MH
222.127	**205.13** *(100)*	9	67		
224.153	85.19 (85.86)	13	67		
224.153	103.12 (18.60)	9	67	6.5–11.5	Cys3MH d_2
224.153	207.13 (100)	7	67		
406.298	162.00 *(88.17)*	22	130		
406.298	**179.2** *(100)*	17	130	7.5–12	G4MMP
406.298	259.51 *(16.08)*	14	130		
408.272	**162.00** *(98.47)*	17	132		
408.272	262.03 *(19.26)*	7	132	9.5–15	G3MH
408.272	279.03 *(100)*	12	132		
410.323	**162.00** *(100)*	20	138		
410.323	264.22 *(94.62)*	15	138	9.5–15	G3MHd_2
410.323	281.14 *(83.32)*	11	138		

Table 3
Influence of DOWEX amount during extraction step.

Compounds	*m/z* Ion (Da)	Average areas of labeled compounds after extraction on DOWEX resin (Arbitrary units, 10^9)		ANOVA ($\alpha = 0.05$)	
		50 mg of DOWEX ($n = 3$)	100 mg of DOWEX ($n = 3$)	*P* value	
Cys4MPP d_6	226	17.7	16.0	0.6526 > 0.05	There is not a statistically significant difference between both extractions.
Cys3MH d_2	224	11.3	7.7	0.1948 > 0.05	
G3MH d_2	410	14.7	10.7	0.3436 > 0.05	

containing their deuterated analogues except for G4MMP which was not available as deuterated compound.

Stock solutions used for the preparation of the calibrating solutions were stocked at −20 °C and their stability was weekly checked over two months by analyzing known amounts and comparing all responses.

Calibration curves were performed by analyzing in duplicate ten aqueous calibrating solutions containing all target compounds at different levels of concentrations of natural compounds and a fixed amount of labeled compounds as reported in Table 1.

2.7. Identification criteria

Cysteinylated and glutathionylated precursors were considered as identified in the samples when meeting the following criteria specified in Commission decision 2002/657/EC [22]:

- The relative retention time of an analyte in a sample and a calibration solution has to be within the ±2.5% tolerance.
- The presence of four identification points (parent ion and three daughter ions (Table 2)).
- The relative ion intensities have to comply with the permitted tolerances (Newmann–Keuls comparison test).

Table 4
Influence of water for the conditioning of C18 cartridges.

Grape variety (Place)	Response increasing (%)			
	G3MH	G4MMP	Cys3MH	Cys4MMP
Melon B. (Nantes)	23%	40%	17%	18%
Sauvignon (Tours)	5%	39%	22%	28%
Sauvignon (Sancerre)	18%	32%	31%	14%
Average	16%	37%	23%	20%

2.8. Validation

2.8.1. Linearity

Both linear and quadratic models were tested performing lack-of-fit test model. Calibration was performed every 5 days using ten calibrating points in duplicate and every time the stability of the model was checked. In addition, blank samples were analyzed to avoid any memory effect on chromatographic system.

2.8.2. Recovery

The overall recovery was determined by spiking three different samples (2 Sauvignon, 1 Melon B) with known amounts of precursors at three levels of concentration for G4MMP (5, 10 and 20 nmol/L) and at one level of concentration for G3MH (10 nmol/L), Cys3MH and Cys4MMP (50 nmol/L). Analyses were performed in triplicate for G4MMP and with 9 repetitions for analytes quantified by SIDA. All added amounts were within the calibrated range. The comparison between spiked and non-spiked values allowed us to calculate the recovery, accuracy and precision for each grape variety. Matrix effect was evaluated according to Matuszewski et al. method [23,24].

2.8.3. Repeatability

The mass spectrometry data obtained for recovery study were also used to determine the repeatability (3 musts with $n = 9$, for

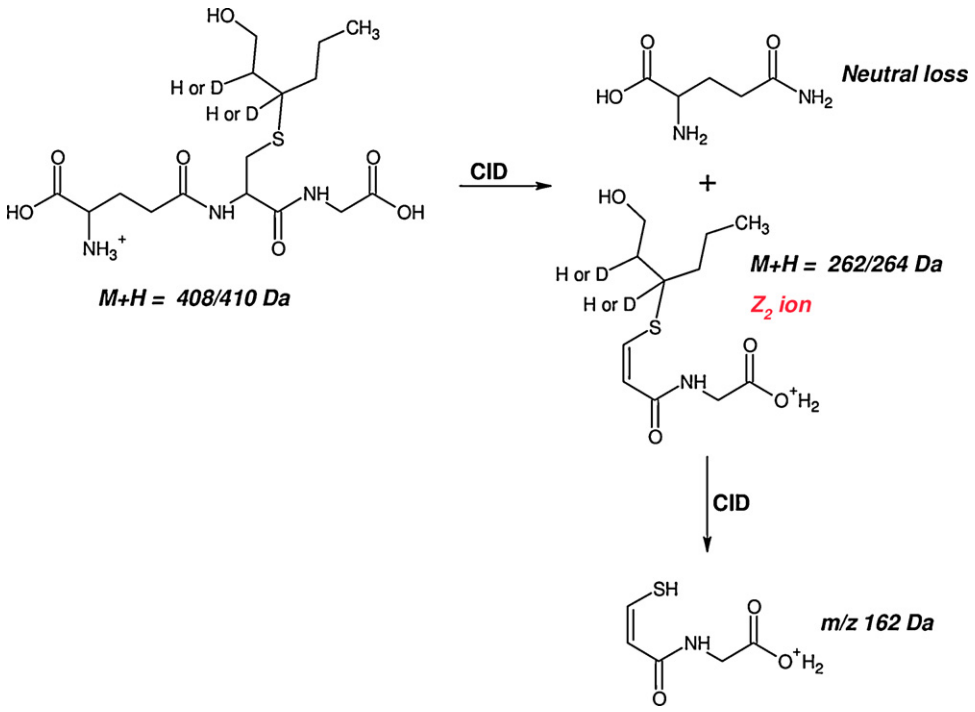

Fig. 2. Fragmentations of G3MH/G3MH d_2 by collision induced dissociation (CID).

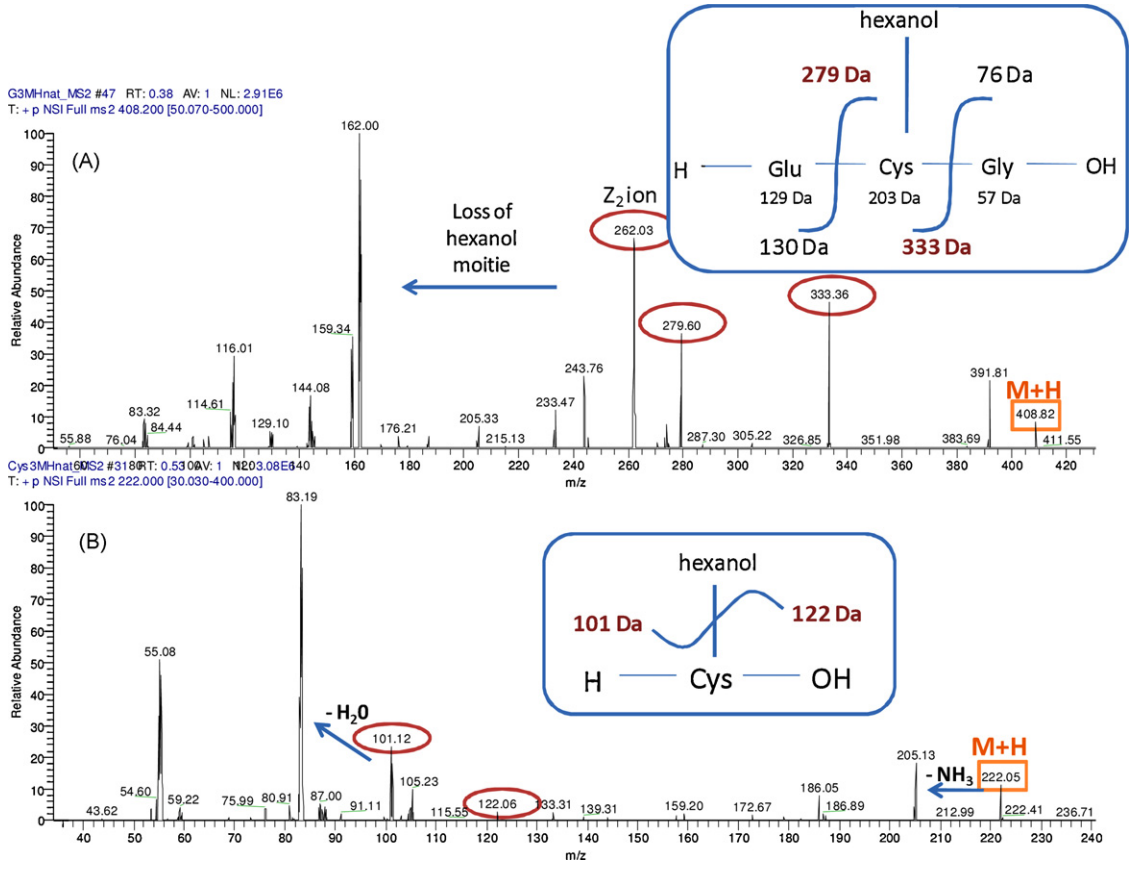

Fig. 3. Fragmentation pattern of G3MH (A) and Cys3MH (B) by infusing standard solutions at 2500 nmol/L into mass spectrometer (Thermo TSQ Vantage).

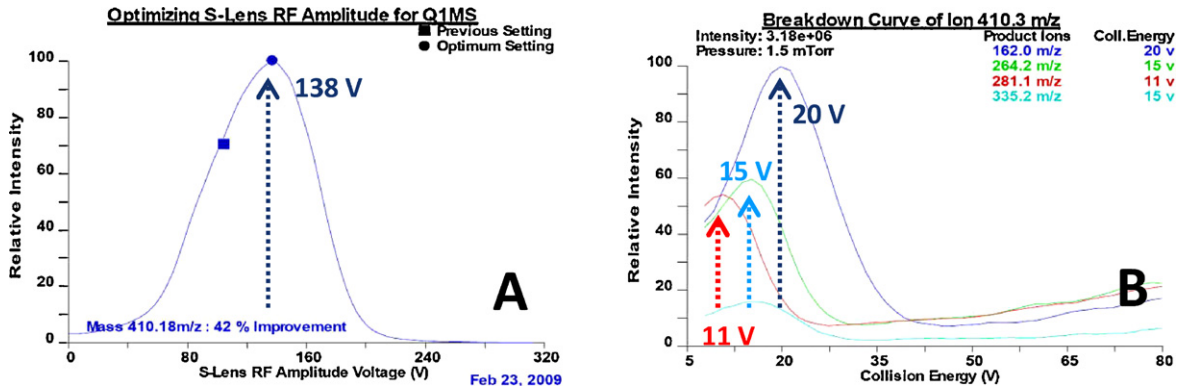

Fig. 4. Example of mass spectrometry parameters optimized for G3MH d_2 (A: S-lens optimization and B: CID optimization) obtained by infusing solution of standards at 2500 nmol/L into triple quadrupole (Thermo TSQ Vantage).

Table 5
Prediction of correlation model using the Lack-of-fit test.

Compounds	P-Value LOF	Model	Equation	Correlation coefficient (R^2)	Range of validity model (nmol/L)
G3MH	0.8733 > 0.01	Quadratic	$Y = -6.1461X^2 + 28.835X - 0.012$	0.9981	0.1–10
	0.1590 > 0.01	Quadratic	$Y = 0.1325X^2 + 1.8301X - 0.0168$	0.9994	
Cys3MH	**0.0006 < 0.01**	**Non-linear**		**0.9978**	**1–100**
G4MMP	0.9776 > 0.01	Linear	$Y = 17396X - 285.225$	0.9871	0.1–10
Cys4MMP	0.2475 > 0.01	Linear	$Y = 0.914X - 0.0268$	0.9973	1–100

analytes quantified by SIDA, and $n = 3$ for G4MMP quantified by external calibration).

2.8.4. Limits of detections and quantifications (LODs and LOQs)

Limits of detection and quantification were evaluated on 18 samples of Sauvignon and Melon B. by measuring the corresponding signal to noise ratio (S/N) for each analytes signal. Limits of detection and quantification were averaged for the whole collection of samples considering S/N = 3 and S/N = 10 respectively [25].

3. Results and discussion

3.1. Mass spectrometer parameters optimization

Target compounds were infused into the mass spectrometer to determine their fragmentation pattern by collision induced dissociation (CID). Glutathione conjugates (G3MH and G4MMP) gave similar fragmentations due to their similar chemical structure. Major fragments were due to the loss of glutamic acid residue (−129 Da), the loss of glycine residue (−75 Da) and to the formation of Z_2 ion by a specific cleavage of glutamic acid residue (−146 Da) (Figs. 2 and 3). Cysteinylated conjugates (Cys3MH and Cys4MMP) gave similar fragmentations resulting from the cleavage of the thioether bond between cysteine and alkyl groups (data not shown).

To perform selected reaction monitoring (SRM) experiments, three daughter ions resulting from parent mass fragmentation were selected for each compound according to their abundance and selectivity.

Optimization of the mass spectrometer parameters that enhance selectivity (adjustment of monoisotopic parent masses) and sensitivity (S-lens and collision energy values) was also performed using the infusion of reference compounds as previously described. The optimized parameters are presented in Table 2 and examples of optimization for sensitivity are shown Fig. 4. S-lens and CID were chosen so that they maximize the intensity of the measured signal, as presented on the graphs. The quantification of such analytes in complex matrixes as musts and at sub μg/L levels required the best sensitivity as possible and the maximization of signal intensity is crucial.

It can be noted that similar S-Lens values were obtained for cysteine conjugates (from 65 to 67 V) and glutathione conjugates (from 130 to 138 V), due to the similarities of the structure. However, no obvious relation can be observed for collision energy.

3.2. Sample preparation optimization

To our knowledge, extraction and purification protocols for both glutathionylated and cysteinylated precursors of 3MH and 4MMP in must were not reported in literature. Consequently, they were adapted from methods already developed for cysteinylated ones.

3.2.1. Dowex extraction

Aroma precursors were extracted from different musts using cation exchange resin Dowex, as this method was already described as being convenient for cysteinylated conjugates [14].

Firstly, the influence of resin amount was studied. Three different grape juices were chosen: one of Melon B. (from Nantes) and two of Sauvignon Blanc (from Tours and Sancerre). Small

Table 6
Recovery yields and matrix effect for G4MMP.

Nominal concentrations (nmol/L)	Mean area of G4MMP (arbitrary units)				Recovery of G4MMP (%)			Matrix effects (%)		
	Neat standard	HAI	Sauv.G	S	HAI	Sauv.G	S	HAI	Sauv.G	S
5	76736	10004	13169	10074	13%	17%	12%	102%	99%	107%
10	157806	19926	31665	18784	13%	20%	12%	100%	99%	103%
20	330931	45776	62785	34990	14%	20%	11%	96%	96%	98%

Table 7
Accuracy measurement.

Grape Variety	Place	% Accuracy (Spiked amounts into musts, $n=9$)		
		G3MH (10 nmol/L)	Cys3MH (50 nmol/L)	Cys4MMP (50 nmol/L)
Melon B.	Nantes	99	116	70
Sauvignon	Tours	90	105	79
Sauvignon	Sancerre	77	102	92
Average		89	108	80

G4MMP spiking (nmol/L) $n=3$ for each level of spiking	Average accuracy at each level (%)	P value ($\alpha=0.05$)	Global accuracy (%)
5	100	0.6599 > 0.05	104
10	107		
20	105		

Table 8
Precision measurement.

Grape Variety	Place	RSD for $n=8$ analysis			
		G3MH (10 nmol/L)	Cys3MH (50 nmol/L)	Cys4MMP (50 nmol/L)	G4MMP (all considered levels of spiking)
Melon B.	Nantes	3%	7%	12%	11%
Sauvignon	Tours	5%	3%	6%	7%
Sauvignon	Sancerre	7%	7%	4%	27%

Table 9
Limits of detection and quantification.

Compounds	Found concentrations in musts* (nmol/L)	S/N	LOD for S/N = 3 (nmol/L)	LOQ for S/N = 10 (nmol/L)
G3MH	23.91	491	0.15	0.50
G4MMP	0.41	53	0.02	0.07
Cys3MH	63.22	132	1.44	4.75
Cys4MMP	7.85	41	0.58	1.91

* Values corresponds to the averaged concentrations measured on eighteen samples.

volumes of each must (600 μL) were spiked with labeled compounds: $Cys3MHd_2$ and $Cys4MMPd_6$ at 1000 nmol/L and $G3MHd_2$ at 100 nmol/L. Extraction was then performed in triplicate on 50 and 100 mg of DOWEX resin. ANOVA test showed a non-significant difference of responses between the two amounts of resin (Table 3). Consequently, further extractions were performed using 50 mg of Dowex.

3.2.2. Purification on C18

Extraction on cation exchange resin allowed the elimination of polyphenols, sugars and other non-charged compounds from must. As a pre-column was used on the nanoliquid chromatograph, direct injection of Dowex extracts was tested. Important interferences were observed under these conditions that considerably decreased the sensitivity.

Thus, a purification step on C18 cartridge was developed, adapted from the protocol proposed for the quantification of S-cysteine conjugates in Petite Arvine musts [15]. Sep-Pak cartridges, convenient for amino-acids purification [26], were chosen and conditioning step was optimized using three different musts. As shown in Table 4, a conditioning with only MeOH, as proposed by Luisier et al. [15], resulted in lower responses than with MeOH follow by water. As a consequence, cartridges were conditionned with MeOH (1 mL) follow by water (2 mL) for all further experiments.

3.2.3. NanoLC separation optimization

Conventional liquid chromatography was used to quantify Cys3MH in Petite Arvine must [15], that is to say the most abundant thiol precursor naturally present in grapes (μg/L levels). To quantify others precursors occurring at lower levels (ng/L levels), it was necessary to use a more sensitive analytical approach. Nanoliquid chromatography seemed to be the method of choice since it provides a better sensitivity, efficiency and shorter analysis times. Indeed, many references in literature proved its efficiency in the field of proteomics, pharmaceutical and environmental analysis [27].

To reach a very good sensitivity, thiol precursors were firstly focused on a pre-column composed by a reverse phase C18 as recommended by Mills et al. [28] and then separated on a similar analytical column. Under these conditions, limits of detection for target compounds were sufficient (inferior to ng/L levels) to be applied for real matrix analysis.

The separation of precursors was based on a classical gradient used in peptidomics [29,30] with acetonitrile and water both slightly acidified with formic acid at 0.1%. Under these conditions, cysteinylated precursors eluted sooner than glutathionylated ones. Even if chromatographic, separation of Cys3MH and Cys4MMP, and, in the same manner, of G3MH and G4MMP, could be optimized, a sufficient selectivity and specificity were reached to distinguish each precursor from the other one using tandem mass-spectrometry in selected reaction monitoring as detection mode. Thus, no further optimization was performed.

Table 10
Repeatability and intermediate precision.

SAMPLES	Concentrations (nmol/L)			
	G3MH	Cys3MH	Cys4MMP	G4MMP
Sauvignon day 1	1.911	14.664	19.171	0.813
Sauvignon day 2	1.984	17.983	15.849	1.115
Sauvignon day 3	2.118	15.566	16.340	0.919
Sauvignon day 4	2.082	18.766	15.350	1.247
Sauvignon day 5	2.049	18.191	15.856	0.993
Mean concentration	2.029	17.034	16.513	1.017
Intermediate precision (%)	**4**	**11**	**9**	**17**
Repeatability (%)	**2** ($n=6$)	**2** ($n=6$)	**5** ($n=6$)	**7** ($n=3$)

Table 11
Identification criteria for G3MH in neat standards (A) and in Melon B. samples (B) (Newmann–Keuls were performed using $\alpha = 0.05$).

(A) Daughter ions	Daughter ions area of G3MH in NEAT STANDARDS (% of base peak)							
	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5	Mean	RSD (%)	Newmann –Keulstest
162.00	97.80	96.86	99.02	99.40	99.25	98.47	1.11%	Group A
262.03	19.00	18.84	18.69	19.58	20.20	19.26	3.24%	
279.03	100.00	100.00	100.00	100.00	100.00	100.0 0	0.00%	
Retention time (min)	11.35	11.35	11.36	11.36	11.36	11.36	0.05%	
(B) Daughter ions	Daughter ions in SAMPLES (% area of G3MH of base peak)							
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Mean	RSD (%)	Newmann–Keulstest
162.00	96.29	98.79	96.44	96.01	95.99	96.70	1.22%	Group A
262.03	19.71	19.69	19.86	19.04	16.93	19.05	6.43%	
279.03	100.00	100.00	100.00	100.00	100.00	100.00	0.00%	
Retention time (min)	11.35	11.36	11.35	11.36	11.35	11.35	0.05%	

3.3. Analytical method validation

3.3.1. Selectivity

Cysteinylated and glutathionylated conjugates of 3MH and 4MMP are naturally occurring in grapes so blank matrix is not available. Nevertheless, our analytical method was based on selected reaction monitoring mode that ensures an accurate selection of parent and daughter ions avoiding most interferences. In the following paragraphs, the measurement of matrix effect and accuracy proved the high selectivity of the method.

3.3.2. Calibration

Calibration for each compound was performed using LC-Quan 2.5 software (Thermo). The calibration curves for isotopic dilution were obtained by plotting the peak area ratio of quantifier ions ($A_{analyte}/A_{standard}$) multiplied by the internal standard concentration versus the corresponding concentration of analytes with 1/X weighting. Calibration curve for G4MMP was obtained by plotting peak area against concentration.

Linearity of these curves was assessed by the lack-of-fit test. The linear model appeared to be adequate for both 4MMP precursors since the P-values for lack-of-fit were greater or equal to $\alpha = 0.01$ (Table 5). However, for G3MH and Cys3MH, the quadratic model was the best regression model even if the Cys3MH calibration curve exhibited a satisfactory correlation coefficient in linear model. Each precursor family exhibited its own correlation model: quadratic for 3MH precursors and linear for 4MMP precursors; independently to the quantification method used (SIDA or external calibration).

3.3.3. Recovery for G4MMP

Quantification of G3MH, Cys3MH and Cys4MMP was performed by stable isotope dilution assay. This technique avoids any recovery studies since natural and labeled compound have similar behaviors. However, quantification of G4MMP by external calibration requires the evaluation of recovery yields because a non-negligible amount of analytes was lost during sample preparation (extraction and purification steps).

G4MMP was spiked into three different musts at three concentration levels (5, 10 and 20 nmol/L) in triplicate in view to evaluate the effect of initial spiked amount on recovery yields. Samples were extracted on cation exchange resin and purified on C18 cartridges followed by nanoLC-MS/MS analysis.

Recovery yields ranged from 11 to 20% as shown in Table 6. The Fisher's least significant difference test proved that recovery yields do not depend on spiking level and grape variety. Thus, it can be assumed that recovery yields are meanly equal to 14%. Even if an important amount of analyte was lost during sample preparation, the method sensitivity was sufficient to accurately quantify G4MMP in must.

3.3.4. Matrix effects

As a blank matrix was not available, matrix effect was evaluated using the same experimental set as for recovery studies. In theory, matrix effects does not affect compounds quantified by stable isotope dilution assay since the suppression or enhancement of ion peak intensity, is similar for natural and labeled analyte. Consequently, we only measured matrix effect for G4MMP which was quantified by external calibration. In practice, peaks areas for neat standard and for samples spiked before extraction and purification steps were compared taking into account the recovery values. If global ratio (area sample/area standard) was inferior or superior to 100%, there is a significant influence of the matrix on analytes measurement [24].

Table 12
Quantification of precursors in Melon B., Sauvignon, Riesling and Gewurztraminer grape juices.

Grape variety Number of samples		Melon B. 36	Sauvignon 21	Riesling 9	Gewurztraminer 5
G3MH concentration (nmol/L)	Mean	<0.5	10.72	3.11	14.62
	Max		3.31	1.67	13.66
	Min		18.44	4.96	17.33
Cys3MH concentration (nmol/L)	Mean	<4.75	101.14	108.59	270.52
	Max		35.92	70.16	263.34
	Min		177.50	138.67	263.20
G4MMP concentration (nmol/L)	Mean	nd	2.21	1.17	0.53
	Max	nd	0.07	1.36	0.25
	Min	nd	10.62	4.53	0.45
Cys4MMP concentration (nmol/L)	Mean	12.23	18.24	<1.9	3.42
	Max	17.45	11.79		2.46
	Min	4.87	28.40		3.72

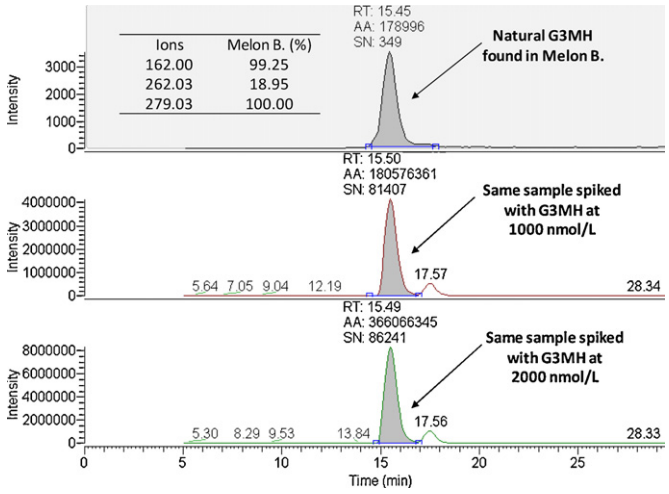

Fig. 5. Identification of G3MH in Melon B. must (Major fragments of synthetic G3MH: 408 Da = >162 Da (98%), 408 Da = >262 Da (18%) and 408 Da = >279 Da (100%)).

As shown in Table 6, no matrix effect is observed in our method as global ratios ranged from 95 to 105% [24]. Consequently, the spiking of known amounts of precursor gave an accurate and precise value. This observation allows performing calibration curves in model solutions instead of real matrix and can be used for quantification on all types of white musts.

3.3.5. Accuracy and precision

Accuracy was evaluated by adding known amounts of synthetic precursors into three different musts at different concentration levels. In parallel, control samples (no spiked musts) were performed to distinguish the natural amount of precursor from the one added. By comparing theoretical spiked amount and calculated amount, we evaluated the accuracy of the method.

Three different musts of Melon B. and Sauvignon Blanc from three different locations (Nantes, Tours, Sancerre) were chosen for this experiment in order to have nine replicates. The G3MH, Cys3MH, and Cys4MMP, quantified by SIDA, were added at one concentration only in these musts (10, 50, and 50 nmol/L, respectively) whereas G4MMP, quantified by external calibration, was spiked at three levels of concentrations (5, 10 and 20 nmol/L). Fisher's least significant difference test was performed to evaluate the influence of grape variety and initial amount spiked on recovery and accuracy.

Extractions and purifications of each sample were performed according to the previous method and analyzed by nanoLC-MS/MS.

Accuracy for all the compounds was satisfactory as averaged value ranged from 80 to 108% (Table 7). The Fisher's least significant difference test showed that accuracy was equivalent independently of the grape variety considered.

The same samples were used to determine the precision. For all compounds, precision was satisfactory since RSD values were inferior or equal to 12% for all data considered (Table 8).

3.3.6. Repeatability and intermediate reproducibility

Repeatability was calculated using one Sauvignon must spiked with known amounts of analytes. Samples were all analyzed the same day with $n = 3$ for G4MMP and $n = 9$ for other compounds. Relative standard deviations ranged from 2 to 7% for all analytes, demonstrating the satisfactory precision of the method (Table 10).

Intermediate reproducibility was evaluated along five consecutive days by extracting, purifying and analyzing the same Sauvignon must each day and by measuring the relative standard deviation of the concentrations obtained (same sample, same analyst and same apparatus). Globally, stable isotope dilution assay provided better reproducibility than external calibration, showing the advantage of using labeled internal standard.

Quantification of all aroma precursors was reproducible with good accuracy and precision over five days (Table 10). Relative standard deviations were quite satisfactory because they were inferior to 11% for quantification by SIDA and inferior to 17% for external quantification.

3.3.7. Limits of detections and quantifications (LODs – LOQs)

Limits of detection and quantification were measured on a set of eighteen must samples. According to the International Conference on Harmonization recommendations, we evaluated limits of detection and quantification using a signal to noise ratio equal to 3 and 10 respectively. Therefore, we measured the average concentration of each precursor in different samples corresponding to a signal to noise ratio equal to 3 and 10. For cysteinylated precursors, LOQs were quite satisfactory (inferior to 4.75 nmol/L) considering levels at which these compounds occur in musts [5,12] (Table 9). For glutathionylated precursors, limits of quantification inferior to 0.5 nmol/L are very convenient to quantify these compounds at traces levels in must (Table 10).

4. Application to natural samples

4.1. Identification of G3MH in Melon B. grape juice

Glutathionylated precursor of 3MH was clearly identified in Melon B. musts using a method that responds to the recommendations specified in the Commission Decision 2002/657/EC [22]. The shift of G3MH retention times between samples and neat standard was inferior to 1% which was in accordance with ±2.5% tolerance. One parent and three daughter ions were selected to identify the analyte in real matrix. Finally, ion ratio comparison between five samples of Melon B. and five neat standards demonstrated the statistical similarity of repartition using the Newmann–Keuls test (Table 11).

Also, the addition of known amounts of synthetic G3MH into extract involved the increase of initial signal, demonstrating that signal resulted from natural G3MH (Fig. 5).

Consistency between retention times and ion ratios in our analyses allowed us to clearly identify glutathionylated precursor of 3MH into Melon B.

4.2. Identification of G4MMP in Riesling and Gewurztraminer grape juices

The identification of glutathionylated precursor of 4MMP was performed using similar strategy as previously developed. The G4MMP was clearly identified into Sauvignon, Riesling and Gewurztraminer musts.

Considering these aspects, we observe that G3MH was ubiquitous whereas the G4MMP seemed to be more specific for certain grape varieties.

4.3. Quantification of precursors in several types of grape juices

Glutathionylated and cysteinylated precursors were quantified into two types of musts: directly prepared in wine cellars for Sauvignon, Riesling and Gewurztraminer and prepared in the laboratory for Melon B. Concentrations of glutathionylated conjugates were systematically lower than those of cysteinylated conjugates (Table 12). Also, G3MH concentrations were more important than those of G4MMP, as already reported for cysteinylated precursors [12].

Recent investigations demonstrated that conversion yield of G3MH into 3MH in Sauvignon Blanc was closed to 4.4%. Assuming this value, the contribution of G3MH for the total production of 3MH could be estimated for all grape varieties from 2 to 86 ng/L. Thus, the G3MH could be considered as another important precursor of 3MH.

5. Conclusion

This is the first analytical method by nanoLC–MS/MS allowing the direct and simultaneous quantification of four precursors of varietal thiols in white grape musts without any derivatization steps. The validation study demonstrated the impressive accuracy, precision and sensitivity of the method allowing the quantification of these compounds at traces levels in grapes. The formal identification of glutathionylated precursors in different grape varieties will bring new insight in the understanding of varietal thiol production in wine.

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