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Identification and Organoleptic Contribution of Vanillylthiol in Wines

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1 Abstract

2 Vanillylthiol, a chemical compound reminiscent of clove and smoke, has been identified for 3 the first time in young red and dry white wines. The chemical structure of this new aroma was confirmed by original chemical synthesis. Vanillylthiol was prepared by two-step procedure 4 from vanillin. The conversion of vanillin to divanilly disulfide was easily achieved by 5 6 treatment with inorganic sulfur-donor reagent. Reduction of the disulfide gave the target thiol 7 in good yield. The quantification of vanillylthiol in wine was performed by non-specific liquid/liquid extraction (CH_2Cl_2), separation of the volatile compounds using gas 8 9 chromatography and specific detection using tandem mass spectrometry (triple quadrupole). 10 Vanillylthiol was found particularly in young wines aged in new oak barrels. These wines 11 contained between a few 50 ng/L to more than 8300 ng/L. The highest levels were found in red wines aged 12 months in new oak barrels. Given its perception threshold in a wine model 12 13 solution (3.8 μ g/L), vanillylthiol may contribute to the spicy, clove-like flavor of red wines 14 aged in oak barrels.

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Keywords: wine, aroma, thiol, oak barrel, aging

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29 INTRODUCTION

30 The use of oak wood is widely recognized as helpful for increasing the intrinsic quality of 31 wines. Contact with wood can occur during winemaking and aging or only during aging or maturation. Indeed, the production of great wines often involves maturation in wood barrels. 32 33 During barrel-aging, wine flavor and taste are considerably modified by compounds released directly from oak wood. Oak non-volatile compounds are non-flavonoid polyphenols that 34 35 include ellagitannins, phenolic acids, coumarins and polymeric compounds, some of which contribute to the perception of astringency and bitterness.¹⁻³ Very recently, lignans such as 36 lyoniresinol have been shown to contribute to the bitterness taste of oak-matured wines.⁴ 37 Moreover, Marchal et al.⁵ identified new triterpenoids extracted from oak wood *Quercus* 38 39 *petraea* which can contribute to the increase in wine sweetness observed during oak aging.⁶ 40 Wines aged in oak barrels are marked by a complex mix of nuances reminiscent of coconut, 41 wood, vanilla, spice and toasted. Although hundreds of oak-derived volatile compounds have 42 been identified and are released during the contact between wine and oak wood, only some of 43 them are really involved in these nuances. For many years, the identification and quantitation 44 of odorant compounds released during barrel aging have received much attention. Vanillin 45 (vanilla), β -methyl-y-octalactone (coconut), volatile phenols (spicy) and 2-furanmethanethiol or furfurylthiol (FFT, toasted) are now considered to be the key molecules associated with 46 oak aging in barrels^{7, 8} and with oak chips.⁹ Volatile substance content is strongly affected by 47 natural factors as well as by botanical species (Quercus robur, Quercus petraea, Quercus 48 49 alba), geographical origins and cooperage techniques such as seasoning and toasting (also called hydrothermolysis). Many other odorants are also associated directly or indirectly with 50 oak wood aging, thus increasing the complexity of wines aged in oak wood barrels.¹⁰⁻¹³ 51

52 These molecules of different chemical nature can be native in oak heartwood or appear during the cooperage process (oak seasoning and toasting). When contact with oak occurs during 53 alcoholic fermentation, yeast metabolism may modify the intensity of "oak wood" flavor.¹⁴ 54 For example, odorant vanillin can be reduced in flavorless vanillic alcohol. On the other hand, 55 veast metabolism gives rise to new very odorant compounds, as evidenced by Blanchard.¹⁵ 56 Following on from Tominaga⁸ who first identified FFT which is reminiscent of coffee in 57 58 wines, he demonstrated that furfural was a possible precursor of FFT. Furfural, which 59 presents a very high perception threshold (Table 1) is released by toasted wood when it comes 60 into contact with wine. In white wines, FFT was present only in barrel-fermented wines. The formation of FFT by yeast in the presence of furfural increased when the conditions were 61 favorable for the production of HS⁻ anions in excess of the amounts required for protein 62 synthesis: high sulfur content (cysteine, sulfates) and relatively low nitrogen levels.¹⁵ 63

64 It is now widely accepted that understanding the contribution of thiol compounds is essential for investigating wine flavor at the molecular level. Indeed, compounds responsible for the 65 fruity character of young Sauvignon Blanc wines and others such as Petite Arvine, Semillon, 66 Petit and Gros Manseng, Koshu, have been identified as volatile thiols.¹⁶⁻¹⁸ The three most 67 important thiols are thought to be 3-sulfanylhexanol (3SH, grapefruit/passion fruit), 3-68 sulfanylhexyl acetate (3SHA, grapefruit/passion fruit) and 4-methyl-4-sulfanylpentan-2-one 69 70 (4MSP, box tree and broom). More recently, other thiols such as 3-sulfanylpentan-1-ol, 3-sulfanylheptan-1-ol which evoke citrus-like note, and the 2-methyl-3-sulfanylbutan-1-ol 71 reminiscent of raw onion, were identified in Sauternes wines.¹⁹ Absent from must, they were 72 73 found in wine after alcoholic fermentation and their concentrations were much higher when Botrytis cinerea had developed on the grapes. 74

Other volatile thiols have also been identified in dry white wines and old champagnes²⁰; ethyl-3-thiopropionate and benzenmethanthiol (smoke). More recently, a new odoriferous thiol associated with an unpleasant odor, ethyl 2-sulfanylacetate (baked beans), was identified in dry wine.²¹ Unlike other varietal thiols, it appears in wine during bottle aging and its concentrations are related to oxygen levels during the pre-fermentative procedures of vinification.

4-Hydroxy-3-methoxybenzenemethanthiol (Vanillylthiol) is a sulfur organic compound (2) described for the first time in 1949 by Kipnis.²² Its structure was validated with no convincing instrumental results compared to the current analytical tools. Moreover, the presence of vanillylthiol in wine and in general in food and beverages has never been reported. This paper describes the synthesis, identification and a methodology for the quantitation of vanillylthiol in red and white wines made with different grapes.

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88 Materials and methods

Chemicals. 4-Hydroxy-3-methoxybenzaldehyde (Vanillin, 98 %) and sodium hydrogen 89 sulfide (98 %) were obtained from Alfa Aesar (Bischheim, France). Anhydrous sodium 90 sulfate (99 %), Ethanol (HPLC grade), dichloromethane (Rectapur grade) diethylether and 91 92 pentane were supplied by Prolabo (France). Lithium aluminum hydride (97 %), thiourea (99 93 %) and 3,4-dimethylphenol (99 %) were purchased from Sigma-Aldrich Chemicals (Lyon, 94 France). All organic solvents and inorganic salts used for organic synthesis were also 95 purchased from Sigma-Aldrich Chemicals (Lyon, France). Acetone-d6 was obtained from Euriso-Top (Saint-Aubin, France). L(+) tartaric acid (99.5 %) was from Fluka (France). 96 97 Tetrahydrofuran was dried by refluxing a solution containing sodium wires and 98 benzophenone under nitrogen and distilled immediately before use. The solution of sodium 99 hydrogen sulfide was prepared with distilled water immediately before use. All moisturesensitive reactions were carried out in an argon atmosphere. 100

101 **Wines.** A Sauvignon Blanc batch from the 2014 vintage was selected for this experiment. 102 After pressing of the grapes (pneumatic press, Vaslin Bucher, France), the must (25 HL) was settled at 200 NTU (nephelometric turbidity unit) and inoculated with Saccharomyces 103 104 cerevisiae Zymaflore X5 strain (100 mg/L; Laffort SA, Floirac, France) and the assimilable 105 nitrogen content was adjusted to 200 mg/L. When one third of the alcoholic fermentation had 106 been completed, the must was placed in three barrels (225 L): two barrels made of new oak, 107 two 1-year-old oak barrels and two 2-year-old oak barrels. The barrels were made from 108 French oak wood (*Quercus petraea*) that was chosen by the cooperage (Seguin Moreau, 109 Cognac) as representative of its production. After the end of alcoholic fermentation, the wines 110 were aged on total lees (7 months). The level of free sulfur dioxide was maintained at 30 111 mg/L during the overall experiment to avoid oxidation and malolactic fermentation. Wine samples were taken from each barrel at the end of alcoholic fermentation and during aging 112 113 where vanillylthiol were assayed.

Moreover, several red and white wines aged or not in oak barrels were provided fromwineries located in several appellations of Bordeaux and Burgundy.

116 Synthesis. Divanillyl disulfide (1). A solution of sodium hydrogen sulfide (10.8 g, 210 mmol) 117 in water (90 mL) was added dropwise to a solution of vanillin (5.0 g, 33 mmol) in ethanol (25 mL) at room temperature and stirred overnight. The mixture was then heated at 60°C for 3h, 118 119 cooled to room temperature and the resulting precipitate was filtered to give the 120 corresponding disulfide in 30 % yield as an odorless white powder (1.62 g; Rf=0.52, Diethyl ether/Pentane: 80/20). FD-MS (+): m/z 338.0647 for $[M]^+$ (calculated for $[C_{16}H_{18}O_4S_2]^+$, 121 122 338.0646) MS (EI, 70 eV) m/z (%) 170 (41), 138 (25), 137 (100), 136 (24), 122 (37), 107 (31), 106 (20), 105 (19), 94 (27), 78 (20), 77 (14), 65 (26), 66 (11). ¹H NMR (Acetone-d6) δ 123 6.88 (d, J = 1.5 Hz, 1H, C<u>H</u>-2), 6.79 (d, J = 8.0 Hz, 1H, C<u>H</u>-5), 6.76 (dd, J = 8.0 Hz, 1.5H, 124 CH-6), 3.84 (s, 3H, CH₃), 3.67 (s, 2H, CH₂). ¹³C NMR (Acetone-d6) δ 148.3 (CH-4), 147.1 125

(<u>C</u>H-3), 129.5 (<u>C</u>H-1), 123.1 (<u>C</u>H-6), 115.7 (<u>C</u>H-5), 113.8 (<u>C</u>H-2), 56.3 (<u>C</u>H₃), 43.7 (<u>C</u>H₂). IR
(solid, KBr) cm⁻¹ 3450 (s), 2979 (m), 1611 (s), 1510 (s), 1463 (s), 1436 (s), 1383 (s), 1368 (s),
1302 (s), 1263 (s), 1233 (s), 1150 (s), 1116 (s), 1028 (s).

Vanillyl thiol (4-Hydroxy-3-methoxybenzenemethanthiol, 2). A solution of divanillyl disulfide 129 130 1 (552 mg, 1.6 mmol) in dry tetrahydrofuran (20 mL) was added to a suspension of lithium aluminum hydride (354 mg, 9.3 mmol) in dry tetrahydrofuran (20 mL) at -78°C under argon 131 132 atmosphere. The reaction was allowed to warm to room temperature for 12 h and then cooled again (ice bath). The reaction was carefully quenched with ice-cold saturated aqueous 133 ammonium chloride solution (40 mL) and pH was adjusted to pH 2-3 with 1 M hydrochloric 134 acid. The resultant mixture was extracted with ethyl acetate (3 x 50 mL). The combined 135 136 organic phases were washed with saturated sodium chloride solution (2 x 50 mL), dried over 137 magnesium sulfate, filtered, and concentrated under reduced pressure to give the title 138 compound as a colorless oil (476 mg, 86 % yield, Rf=0.72, Diethyl ether/Pentane: 80/20). The purity of the compound obtained, which was determined by GC-MS and 1H NMR, was > 95 %. 139 This compound was thus used without purification. FD-MS (+): m/z 170.0408 for $[M]^+$ 140 (calculated for $[C_8H_{10}O_2S]^+$, 170.0401). MS (EI, 70 eV) m/z (%) 170 (17), 137 (100), 138 141 (10), 122 (16), 107 (2), 94 (9), 78 (3), 65 (5), 66 (2). ¹H NMR (Acetone-d6) δ 7.51 (s, 1H, 142 143 OH), 6.95 (d, J = 1.4 Hz, 1H, CH-2), 6.79 (dd, J = 8.0, 1.4 Hz, 1H, CH-6), 6.75 (d, J = 8.0144 Hz, 1H, CH-5), 3.83 (s, 3H, CH₃), 3.69 (d, *J* = 7.4 Hz, 2H, CH₂), 2.10 (t, *J* = 7.4 Hz, 1H, SH). ¹³C NMR (Acetone-d6) δ 148.2 (CH-3), 146.3 (CH-4), 133.8 (CH-1), 121.4 (CH-6), 115.6 145 (CH-5), 112.5 (CH-2), 56.2 (CH₃), 28.9 (CH₂). IR (liquid) cm⁻¹ 3436 (s), 2963 (m), 2937 (s), 146 147 2842 (s), 2563 (s), 1726 (s), 1606 (s), 1513 (s), 1463 (s), 1432 (s), 1370 (s), 1271 (s), 1232 (s), 1152 (s), 1121 (s), 1032 (s). 148 Reaction of vanillyl alcohol with thiourea.²³ A solution of vanillyl alcohol **3** (2.87 g, 18.6 149

150 mmol) in dry tetrahydrofuran (20 mL) was added to a solution of thiourea (1.5 g, 19.7 mmol)

in 2 M hydrochloric acid (15 mL) at room temperature. The reaction was heated at 55 °C for 1 151 152 h and stirred at room temperature for 12 h. The resulting mixture was then treated with a 3 M NaOH solution (11 mL) and heated at 50 °C for 4 h. The reaction mixture was cooled to 153 room temperature and then extracted with diethyl ether (3 x 50 mL). The pooled organic 154 155 phases were washed with brine (3 x 50 mL), dried over magnesium sulfate, and filtered. The 156 solvent was removed in vacuo to give a mixture of four compounds as a yellow oil (1.2 g). 157 The crude was purified by flash chromatography by using a polarity elution gradient 158 (Pentane/Et₂O: 7/3 to 4/6). Divanillyl sulfide 4 (white solid, 0.584 g, 44 % yield, Rf = 0.54, Diethyl ether/Pentane: 159 80/20). FD-MS (+): m/z 306.0934 for $[M]^+$ (calculated for $[C_{16}H_{18}O_4S]^+$, 306.0925). MS (EI, 160 70 eV) m/z (%) 306 (40), 170 (25), 138 (53), 137 (100), 122 (29), 107 (23), 94 (19), 78 (10), 161 65 (14). ESI (-): $[M-H]^{-} = 305.1$; TOF (+): $[M+Na]^{+} = 329.1$; FD-MS (+): $[M]^{+} = 306.1$. ¹H 162 163 NMR (Acetone-d6) δ 7.48 (s, 1H, OH), 6.89 (s, 1H, CH-2), 6.76 (m, 2H, CH-5, CH-6), 3.82 (s, 3H, CH₃), 3.59 (s, 2H, , CH₂). ¹³C NMR (Acetone-d6) δ 148.3 (CH-4), 146.5 (CH-3), 164 130.7 (CH-1), 122.5 (CH-6), 115.4 (CH-5), 113.2 (CH-2), 56.2 (CH₃), 36.2 (CH₂). IR (solid, 165 KBr) cm⁻¹ 3497 (s), 3439 (s), 3061 (m), 3034 (m), 2983 (s), 2950 (s), 2917 (s), 2843 (s), 1605 166 (s), 1513 (s), 1460 (s), 1446 (s), 1428 (s), 1358 (s), 1267 (s), 1227 (s), 1206 (s), 1157 (s), 167 1140 (s), 1113 (s), 1020 (s). 168 Bis(4-hydroxy-3-methoxyphenyl)methane 5 (white solid, 0.284 g, 15 % yield, Rf = 0.55, 169

Diethyl ether/Pentane: 80/20). FD-MS (+): m/z 260.284 for $[M]^+$ (calculated for $[C_{15}H_{16}O_4]^+$, 170

260.2851). MS (EI, 70 eV) m/z (%) 261 (62), 260 (100), 243 (40), 229 (100), 213 (30), 197

171

(20), 185 (28), 157 (18), 137 (50), 124 (25), 107 (23), 94 (19), 77 (14), 65 (14). ¹H NMR 172

(Acetone-d6) δ 7.34 (s, 2H, O<u>H</u>), 6.82 (d, J = 1.8 Hz, 2H, C<u>H</u>-2), 6.73 (d, J = 8.0 Hz, 2H, C<u>H</u>-173

5), 6.65 (dd, J = 8.0, 1.8 Hz, 2H, CH-6), 3.78 (s, 8H, CH₃, CH₂). ¹³C NMR (Acetone-d6) δ 174

148.2 (CH-4), 145.6 (CH-3), 134.2 (CH-1), 122.0 (CH-6), 115.5 (CH-5), 113.2 (CH-2), 56.2 175

(CH₃), 41.6 (CH₂). IR (solid, KBr) cm⁻¹ 3433 (s), 3052 (m), 3027 (m), 2961 (s), 2936 (s), 176 177 2904 (s), 2836 (m), 1604 (s), 1515 (s), 1454 (s), 1427 (s), 1374 (s), 1318 (m), 1265 (s), 1233 178 (s), 1206 (s), 1183 (s), 1142 (s), 1114 (s), 1035 (s), 1018 (s). 179 **Extraction.** Solvent extraction. 50 mL of wine sample were spiked with 50 μ L of 3,4dimethylphenol (10 mg/L, EtOH) as internal standard. The wines were also extracted at room 180 181 temperature with 10 mL of CH₂Cl₂ followed by 5 mL of CH₂Cl₂ under magnetic stirring (10 and 5 min at 700 rpm, respectively). Organic phases obtained were blended, dried over 182 183 anhydrous sodium sulfate, and concentrated to 500 μ L under a nitrogen stream. The sample

184 was stable 5 days at -20° C.

185 SPME experiments. 65 µm SPME fibers coated with Carboxen/Polydimethylsiloxane 186 (Carboxen/PDMS, StableFlex, Supelco) and Divinylbenzene/Carboxen/ Polydimethylsiloxane (DVB/Carboxen/PDMS, StableFlex, Supelco) were used, and the extraction procedure was 187 188 carried out in the gas phase of the SPME vial without immersion in the sample solution, i.e., 189 by HS-SPME method. During analysis of vanillylthiol by SPME, 10 mL of the wine sample 190 was injected into a 20 mL SPME vial. The SPME efficiency was evaluated using the 191 following parameters: (a) extraction temperature, 40 and 60 $^{\circ}$ C; (b) extraction time, 20 and 50 192 min; (c) ammonium sulfate addition, 0 and 5 g; (d) dilution factor 1 and 10.

Instrumentation. The chromatographic system comprised a gas chromatograph Trace GC
Ultra (Thermo Electron, USA) coupled to a triple quadrupole mass spectrometer TSQ
Quantum XLS operated in EI mode. The GC system was equipped with a Triplus RSH autosampler.

197 *Gas chromatography method.* GC separation was carried out on a non-polar ZB-1MS 198 capillary column (100 % polydimethylsiloxane, 60 m; 0.25 mm; 1 μ m) from Phenomenex 199 (France). The carrier gas was He (Linde gas, Bordeaux), 5.3 grade, with a flow rate of 1 200 mL/min. A 1 μ L sample was injected via the autosampler into a split/splitless programmable-

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temperature injector (closure time: 1 min, split flow 30 mL/min) and set as follows: 0.5 min at
230 °C and increasing to 250 °C at 14 °C/min and kept at that temperature for 10 min. Oven
temperature was initially set at 50 °C, held for 1 min, then increased to 160 °C at 6 °C/min,
increased to 222 °C at 3 °C/min and finally kept at 260 °C at 20 °C/min for 10 min. The MS
transfer line temperature was set at 250 °C. *Mass spectrometry.* The mass spectrometer was used in electron impact mode (EI). Source

parameters were optimized as follows: source temperature, electron energy and emission current were set at 230 °C, 70 eV and 30 μ A, respectively. The mass spectrometer was operated in Selected Reaction Monitoring (SRM) mode using argon as collision gas in cell collision (1.5 mTorr). SRM conditions were optimized, as well as collision energy and gas pressure values applied to each precursor ion and were dependent on each transition. PFTBA (Perfluorotri-*n*-butylamine) was used for mass calibration. Data acquisition and analyses were performed using the Xcalibur software version 2.1 supplied by the manufacturer.

214 To determine the retention times of the analytes and their characteristic mass fragments, the 215 primary EI mass spectra were recorded in full-scan mode (m/z 45–250). For selected reaction 216 monitoring (SRM) experiments, precursor ions were selected according to abundance and 217 product ion scans were investigated using the energy ramp function from 5 to 25 V. We also repeated the analysis at different argon collision gas pressures (0.5, 1, 1.5 mTorr). SRM 218 219 conditions were set to a mass resolution of full width at half maximum height (FWHM) in Q1 220 and Q3 with a value of 0.7 Da and a scan time of 0.1 s. Collision energy was systematically 221 set to 10 V. The transitions were chosen with respect to optimized signal-to-noise (S/N) ratios 222 as well as absolute ion intensities for a standard solution of the analytes at 50 μ g/L. Vanillylthiol was monitored at m/z 170 \rightarrow 137 (quantifier) and m/z 137 \rightarrow 94 and 137 \rightarrow 223 224 122 (qualifier). As the internal standard was detected in selected ion monitoring (SIM, m/z =107), analyses were performed in two segments: IS (seg. 1), vanillylthiol (seg. 2). For 225

quantitation, peak area ratios of the analytes and the IS were calculated as a function of the 226 concentration of the substances. Linear retention indices (LRI) were obtained by simultaneous 227 injection of samples and a series of alkanes (C_7-C_{23}) , detection was performed in scan mode.²⁴ 228 Validation of the method. The calibration curves were established between 0.02 and 20 μ g/L 229 of vanillylthiol in a white wine (8 points). The limit of detection (LOD) was defined as the 230 231 concentration which gave a signal-to-noise ratio (S/N) of 3. The limit of quantitation (LOQ) 232 was defined as the concentration which gave an S/N ratio of 10. These parameters were 233 experimentally calculated from the S/N obtained in wine sample analyses. Accuracy of the 234 method was assessed using replicates spiked at one level. Five replicate analyses were 235 performed for the determination of relative standard deviation (RSD).

236 **Determination of detection thresholds.** The detection threshold of vanillylthiol in model solution (12 % ethanol, 5 g/L tartaric acid, pH 3.5) and wines was determined by using a 237 three-alternative forced choice presentation (3-AFC). The solutions were presented in glasses 238 239 corresponding to AFNOR (Association Française des Normes) standards. The odor detection threshold corresponded to the minimum concentration below which 50 % the tasters 240 statistically failed to recognize the difference from the control.²⁵ The sensory panel consisted 241 of 20 students and researchers (half of male and female) between 20 and 40 years old, from 242 the ISVV. 243

General features. Magnetic Resonance Spectroscopy (NMR): ¹H and ¹³C NMR spectra were
recorded on a Bruker AC-300 FT (¹H: 300.13 MHz, ¹³C: 75.4 MHz), spectra referenced using
the lock frequency of deuterated solvent. Chemical shifts (δ) and coupling constants (J) are
expressed in ppm and Hz, respectively. Infrared (IR) spectra were recorded with a Perkin–
Elmer paragon 1000 FT-IR spectrophotometer in the 4000-600 cm⁻¹ range. Field desorption
mass spectra (FD-MS) were performed by the CESAMO (University of Bordeaux, France).
The measurements were carried out on a TOF mass spectrometer AccuTOF GCv by JEOL

251 (Croissy sur Seine, France) using an FD emitter with an emission voltage of 10 kV. One to 252 two microliters of the compound solution in methanol were deposited on a 13-mm emitter 253 wire. Thin-layer chromatography (TLC) was performed on 60F TLC plates: thickness 0.25 254 mm, particle size 10 μ m, pore size 60 Å. Merck silica gel 60 (70–230 mesh and 0.063–0.200 255 mm) was used for flash chromatography. Spots were revealed with UV as well as with 256 sodium nitroprussiate (1.5% in MeOH/NH₃/HCl).

257 **Results and discussion**

258 **Chemical synthesis.** Vanillylthiol was mentioned in 1949 by Kipnis *et al.*²², who studied the 259 preparation of aromatic and heterocyclic thiols by the interactions of aldehydes with hydrogen 260 disulfide. This was the only time when vanillylthiol was reported and it was characterized 261 simply by elemental analysis and refraction index, methods that are clearly insufficient for 262 strict structural identification.

263 In the current literature, thiols are classically synthesized in two steps *via* the formation of a reactional intermediate either by addition to a multiple bond of thiol derivatives (RSH, ArSH, 264 RCOSH)^{19, 26-28} or by substitution with a sulfur containing nucleophiles.^{23, 29-31} Thioacetic acid 265 266 and potassium thioacetate are the most popular sulfur reagents used for the formation of stable thioester intermediates, which can be easily isolated and purified before the next step. Target 267 thiols can then be obtained in good yield by mild saponification or reduction.^{30, 31} 268 269 Alternatively, thiourea can also be used as an odorless sulfur reagent to convert alcohols to the corresponding thiols *via* the basic hydrolysis of the unstable isothiouronium salt.²³ 270

As a single procedure described in literature for vanillylthiol preparation cannot be considered as safe and environmentally friendly,²² using thiourea as sulfur atom source in reaction with vanillyl alcohol **3**, which was obtained by the reduction of vanillin with sodium borohydride in methanol, as previously described by Feng,³² was chosen as the first approach (Figure 1). In our case, the target thiol vanillylthiol **2** and its corresponding disulfide **1** were obtained

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276 only at trace amounts. The major disadvantage of this approach was the formation of a 277 number coproducts, mainly divanillyl bis(4-hydroxy-3of sulfide 4 and methoxyphenyl)methane 5. Compound 4 probably resulted from desulfurization of 1 or from 278 C-sulfanylation of **3** by thiol **2**.³³ The presence of **5** can be explained by the self-condensation 279 of **3**.³⁴ Interestingly, our result differed from that of Kofod, who used thiourea for direct one-280 pot conversion of furfuryl alcohol to the corresponding thiol,²³ and from that of Firouzabadi, 281 who used thiourea to synthesize disulfides from halides.³⁵ The identification and the structural 282 characterization of all coproducts became possible only after the meticulous purification of 283 the crude by flash chromatography using a polarity gradient. All attempts to convert isolated 284 285 divanilly sulfide 4 to vanilly thiol using various reducing agents were unsuccessful.

Since a simpler procedure was needed, vanillin was directly treated by inorganic H_2S donors, sodium hydrogen sulfide (NaSH), in hydro-alcoholic solution under gentle heating. The formation of a precipitate indicated the progress of the reaction. In these eco-friendly conditions, divanillyl disulfide **1** was produced cleanly. Furthermore, no work-up or purification procedures were needed and it was isolated in 30 % yield by simple filtration of the reaction mixture. The disulfide **1** was then easily transformed into the target thiol **2** using lithium aluminum hydride as reducing agent.

Identifying vanillylthiol in wine by GC-MS/MS. We first injected the pure compound in 293 order to determine its retention time and its linear retention indice (LRInon polar 1495) as well as 294 295 its mass fragmentation (Figure 2) in which we could observe the characteristic fragmentation 296 of thiol, with the loss of -SH [M-33]. Volatile compounds of a young white wine and a red 297 wine both aged in oak barrels were extracted with CH₂Cl₂ and analyzed first by GC-MS/MS 298 in scan, SIM and finally in SRM mode. In white wine, a peak corresponding with the same 299 retention time (Rt 37,65 min) and the same mass fragmentation as that of pure vanillylthiol was observed, thereby validating its identification (result not shown). A clean mass spectrum 300

301 was not obtained in red wine owing to coelution, so its identification in scan (EI) mode was 302 not possible. Indeed, as depicted in Figure 3, a small overlapping peak was observed at 37.65 min for ion m/z 137, but no clear peak was detected at m/z 170. For this reason we decided to 303 304 validate its identification using an MS/MS, optimized first with pure vanillylthiol and using two transitions: $m/z \ 170 \rightarrow 137$ and $m/z \ 137 \rightarrow 120+94$ (Figure 4). GC-MS/MS demonstrated 305 306 the existence of vanillylthiol in the same red wine extract with a peak at the retention time of 307 vanillylthiol (Figure 5). Furthermore, whatever the transition chosen, the peak shape formed 308 by the selected ions was not modified by coinjection of a comparable quantity of the reference 309 compound. To our knowledge, this is the first time that this new volatile thiol has been 310 reported in nature and consequently in wines.

Sensorial considerations. The olfactory impact of this new thiol on the overall aroma of wines was examined by determining its odor threshold. Both in pure and diluted form (50 μ g/L, 12 % EtOH), it smells clove-like with smoky notes (Table 1). As for other thiols found in wines and resulting from the biotransformation of aldehydes, the presence of the –SH function considerably modifies their organoleptic properties.^{8, 36}

To illustrate this phenomenon, Table 1 shows the perception threshold as well as the common descriptors of aldehydes and their thiol equivalents found in wines. In our case, the presence of the –SH group in the vanillin structure gives rise to a more intense aroma with new sensorial properties, ranging from a slightly intense vanilla flavor for vanillin to a more intense odor of clove and smoke for vanillylthiol with a perception threshold which was lower by the factor of 15.

Although various hypotheses have been proposed to explain some of these effects,³⁷ the mechanistic interpretation of the variability in odor perception threshold is still rather uncertain owing to the multi-step processes involved in olfaction: odorant diffusion in the

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mucus layer, effect of odorant-binding proteins, biotransformation enzymes, and interactionwith a large repertoire of olfactory receptors.

Assay of vanillylthiol in wines. The determination of thiol compounds at low concentrations 327 328 in wines is known to be tricky owing to their propensity to undergo oxidative reactions during extraction and pre-concentration, as well as to their highly fragmented mass spectrum (EI 329 330 mode) which leads to low inherent sensitivity and/or matrix effects. To overcome these 331 drawbacks, several means to remove matrix components and concentrate the sample have been proposed.^{17, 39} The original method of Tominaga et al.¹⁷ was based upon the selective 332 333 extraction of thiols from an organic extract of wine using the reversible chelation between the SH function and sodium *p*-hydroxymercuribenzoate. Later, other studies focused on sample 334 preparation development or derivatization.⁴⁰⁻⁴² 335

As quantitation of thiol compounds might be tricky with conventional GC-MS approaches 336 337 according to the compound, we decided to use a more sensitive apparatus based on GC-MS/MS triple quadrupole technology. This approach is widely used when the assay is 338 339 performed on LC-MS/MS, but very few studies have reported its use for quantitating odorants in wines. However, Langen⁴³ reported the usefulness of tandem mass spectrometry in the 340 specific detection of γ - and δ -lactones and Mayr⁴⁴ recently reported a similar approach for 341 quantitating oxidation-related flavors including aldehydes, ketones, furans and some alcohols. 342 Very recently, Thibon⁴⁵ used this approach to assay at the same time thiols and oxidation-343 344 related compounds in white wine with LOD close to ng/L.

Two approaches were evaluated for the extraction of vanillylthiol from wine: liquid/liquid extraction and solid phase microextraction (SPME). Two fibers with different coatings of DVB/Carboxen/PDMS and Carboxen/PDMS and several preparation conditions including the choice of temperature, extraction time, salt addition, and dilution factor of the wine were tested. Unfortunately, whatever the conditions, the SPME technique was not able to extract

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this polar organic compound from the wine matrix. On the contrary, our preliminary experiments showed that the liquid/liquid extraction technique with CH_2Cl_2 as solvent was very suitable.

To optimize SRM detection, we had to consider the available mass fragments of vanillylthiol before starting fragmentation. In the mass spectra, intense (base) peaks at m/z 137 $[M - SH]^+$ or 170 M⁺ are the predominant signals (Figure 2). To evaluate the SRM experiments for vanillylthiol analysis, we chose the transition m/z 170 \rightarrow 137, which presents a higher signal intensity compared to the other transition $137 \rightarrow 122+94$ (Figure 4).

358 Finally, the repeatability of the assay was confirmed by a series of five extractions of a non 359 wooded sample of a white wine spiked with 500 ng/L of vanillylthiol. Precision estimated in 360 terms of (RSD) was 9 %. The LOD and LOQ of the method were 24 ng/L and 76 ng/L, respectively. The linearity of the method was determined by plotting the calibration curves of 361 362 the corresponding transition peak areas, normalized by that of the internal standard. In the concentration range (LOQ-5000 ng/L), the control curve was linear ([Vanillylthiol (ng/L] = 363 0.334 H/Hei, $R^2 = 0.995$). This standard curve was measured using the height of the peaks 364 rather than the peak areas because the chromatogram showed a small contaminated peak just 365 366 after the vanillylthiol (Figure 5).

Table 2 shows the results obtained for the vanillylthiol assay in six dry white wines and six 367 368 red wines aged or not in oak barrels, from different appellations, vines and from several 369 vintages and bottle aging. The levels found varied according to the type of wine, the age, and 370 mainly according to the aging period in oak barrels. According to the sample analyzed, white 371 wines generally contained lower levels of vanillylthiol than red wines. The odor activity value (OAV) of vanillylthiol in these wines is systematically <1. The wines kept a long time in 372 373 barrels had the highest concentrations. The highest level of 8394 ng/L (OAV 2.2) was found in a young merlot red wine (2013 vintage) kept for 12 months in oak barrels and analyzed at 374

that time. It is therefore very likely that this thiol may contribute to the clove-spicy flavor of this red wine. For aged wines the levels were low (< 100 ng/L). This result is not surprising owing to the high instability of thiol compounds during aging in bottles.^{21, 46}

Incidence of barrel aging on vanillylthiol formation. Table 3 shows the evolution of 378 vanillylthiol concentrations in a white wine aged in different barrels. As can be seen, the 379 380 vanillylthiol level was strongly affected by the container used. The wines fermented and kept 381 with their lees in new oak barrels had a higher content than those fermented in used barrels. 382 Previous work on the formation of FFT in wine fermented and aged in barrels can shed light on how vanillylthiol can be formed in wine. Blanchard¹⁵ found that high levels of furfural 383 384 released by toasted oak wood during aging were associated with the formation of FFT. The 385 substitution of the SH group for the furfural carbonyl group can be biochemical during alcoholic fermentation (Saccharomyces cerevisiae) or malolactic fermentation (Oenococcus 386 oeni) and chemical during barrel aging, but it always occurs in the presence of H₂S.⁴⁷ 387 388 Therefore, vanilly thiol might be formed in the same way but from vanillin, which is also released by toasted oak wood. As vanillin accumulation is strongly linked with cooper 389 seasoning procedures,⁴⁸ aging time and the volume of the barrel⁴⁹ and heating intensity,⁵⁰ 390 391 vanillylthiol concentration might be modulated by this parameter.

Finally, given its odor properties and its levels found in wines, we postulate that it might 392 393 contribute to the spicy, clove-like flavor of wine, and particularly red wine, aged for a long 394 time in new oak barrels. Or course, further works will be necessary to have a better overview 395 of its distribution in wine and to understand its contribution to the overall aroma of wines aged in oak barrels, this work is in progress. Moreover, it cannot be excluded that this new 396 thiol might contribute to the spicy flavor of wines in combination with other volatile 397 compounds reminiscent of similar flavors and extracted from oak wood such as eugenol and 398 isoeugenol. 399

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- 527 Figure 1: Chemical pathways studied for vanillylthiol synthesis
- 528 Figure 2: Fragmentation spectrum of vanillylthiol under GC-MS in scan mode (EI, 70 eV)
- 529 Figure 3: Example of a chromatogram corresponding to the analysis by GC/QqQ-MS in SIM
- mode of liquid/liquid extract of a red wine for two selected ions: m/z 170 and m/z 137. The
- retention time of vanillylthiol is indicated by an arrow
- 532 Figure 4: Product ion scan of m/z 136.8 (A) and 169.7 (B) obtained from vanillylthiol
- 533 fragmentation under GC/QqQ-MS
- 534 Figure 5: Detection of vanillylthiol and verification of peak purity by co-injection of a
- comparable quantity of the reference compound in a merlot red wine (Medoc appellation,
- 536 2014 vintage) by GC-MS/MS in SRM mode by overlapping ions selected corresponding to
- two transitions $170 \rightarrow 137$ (A); $137 \rightarrow 122+94$ (B), at the retention time of the reference
- 538 compound indicated by an arrow.
- 539
- 540

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Table 1: Example of perception thresholds and descriptors of important thiols and their corresponding aldehydes identified in wines

Compounds	Perception threshold (μ g/L)	Descriptors
Benzaldehyde	3000 38	Almond
Benzenemethanthiol	0.00003 36	Gun flint
Furfural	15000 14	Roasted
Furfurylthiol (FFT)	0.00004 ⁸	Roasted bean, coffee
Vanillin	65 ¹⁴	Vanilla
Vanillylthiol (VSH)	3.8	Clove, smoke

Color	Vine	Appellation	Vintage	Barrel aging period (months)	Bottle aging period (year)	Vanillylthiol (ng/L)	OAV
W	Sauvignon	Bordeaux	2014	0	0	< LOQ	<1
W	Sauvignon	Pessac Leognan	2013	8	1	83 (22)	<1
W	Sauvignon	Pessac Leognan	2013	8	0	818 (38)	<1
W	Semillon	Pessac Leognan	2013	8	0	420 (28)	<1
W	Chardonnay	Burgundy	2013	10	0	1006 (59)	<1
W	Chardonnay	Burgundy	2013	10	0	1426 (89)	<1
R	Merlot	Pessac Leognan	2013	12	0	8394 (198)	2.2
R	Cabernet Sauvignon	Medoc	2013	10	0.5	867 (44)	<1
R	Merlot	Medoc	2014	6	0	1022 (31)	<1
R	Cabernet Sauvignon	Pessac Leognan	2014	6	0	2011 (122)	<1
R	Merlot	Saint Emilion	2010	12	3.5	< LOQ	<1
R	Merlot	Saint Emilion	2010	12	3.5	< LOQ	<1

Table 2: Vanillylthiol concentrations (ng/L) in young and old wines from various appellations and vines. n = 2.

	Must	Wines (aging period)		
		3 months	7 months	
New Barrels	nd	< LOQ	422 (32)	
1.y Barrels	nd	< LOQ	231 (28)	
2.y Barrels	nd	< LOQ	198 (34)	

Table 3: Evolution of vanyllylthiol concentrations in a Sauvignon Blanc wine during barrel aging. n=2

nd: not detected













Time (min)

Figure 4







TOC graphic

