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## Methyl salicylate, a grape and wine chemical marker and sensory contributor in wines elaborated from grapes affected or not by cryptogamic diseases

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1 **Methyl salicylate, a grape and wine chemical marker and sensory**  
2 **contributor in wines elaborated from grapes affected or not by cryptogamic**  
3 **diseases**

4  
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29 **ABSTRACT.**

30 Methyl salicylate (MeSA) is a plant metabolite that induces plant defence resistance and an  
31 odorous volatile compound presenting green nuances. This volatile compound was shown to  
32 be present in wine samples, sometimes at concentrations above its olfactory detection  
33 threshold. MeSA is localized in grapes, particularly in the skins and stems, and is extracted  
34 during red wine vinification. It was detected at the highest concentrations in wines of several  
35 grape varieties, made from grapes affected by cryptogamic diseases, namely downy mildew  
36 caused by *Plasmopara viticola*, and black rot caused by *Guignardia bidwellii*. It has also been  
37 detected in wines from vines affected by Esca, a Grapevine Trunk Disease. MeSA can also be  
38 considered to be a chemical marker in grapes and wine indicative of the level of development  
39 of several vine cryptogamic diseases.

40 **KEYWORDS.** Methyl salicylate, Green aromas, Red wines, Stem, Cryptogamic diseases,  
41 Downy mildew, Grape black rot, Grapevine Trunk Disease (Esca)

42

## 43 **1. Introduction**

44 Methyl salicylate (MeSA) is a volatile odorous compound presenting green and mint-  
45 like flavour nuances. This compound is a secondary plant metabolite, and the main  
46 constituent of essential oils from the shrub genus *Gaultheria*, in particular, the oil of  
47 *Gaultheria procumbens* (98 %) (Gurung, 2007), which is known as “wintergreen”. Its fragrant  
48 properties are appreciated and commonly used as an aroma in chewing gum, sweets and  
49 dental products. It is also known to possess analgesic and antipyretic properties (Chan, 1996).  
50 Methyl salicylate is widespread in plants and vegetables. For example, previous studies have  
51 shown it to be an odorous compound associated with green pepper and tomato aromas and  
52 have demonstrated that it is synthesized from salicylic acid by salicylate methyl transferase  
53 (Buttery et al., 1969; Buttery et al., 1990; Tieman et al., 2010).

54 Its involvement in plant defence phenomena has been highlighted. MeSA is an  
55 herbivore-induced plant volatile (HIPV) (Gadino et al., 2012). This compound is released by  
56 a number of plants (e.g., beans, hops, tomatoes, cucumbers, peppers) in response to herbivore  
57 attack, as in the case of grapevines with leaves affected by spider mite (*Tetranychus urticae*)  
58 infestation (Van Den Boom et al., 2004). The action of MeSA was shown to have (i) a direct  
59 insecticidal effect, for example on spotted-wing drosophila (*Drosophila suzukii*) (Kim et al.,  
60 2016) and (ii) an indirect attracting effect for several natural predators of spider mites on hops  
61 (*Chrysopa nigricornis*, *Hemerobius sp.*, *Deraeocoris brevis*, *Stethorus punctum*) (James &  
62 Price, 2004). Similar effects were observed with natural enemies of aphids on soybeans, such  
63 as seven-spotted ladybugs (*Coccinella septempunctata*) (Mallinger et al., 2011; Zhu & Park,  
64 2005). In addition, MeSA plays a key role in the induction of systemic acquired resistance  
65 (SAR) in plants (Tang et al., 2015). In particular, it was established that MeSA was  
66 synthesized from salicylic acid by tobacco plants infected with tobacco mosaic virus (Park et  
67 al., 2007). Its release into the air constitutes a volatile defence signal that activates the

68 resistance of neighbouring tobacco plants (Shulaev et al., 1997). Karban et al. (2014), who  
69 combined the results of 48 studies, confirmed the existence of "chemical communication"  
70 between plants. MeSA is therefore an indicator of diseased plants (Jansen et al. 2015) and has  
71 been evidenced as biomarker of grapevine leaves infected with downy mildew caused by  
72 *Plasmopara viticola* (Chalal et al., 2015).

73 In grapes and wine, MeSA was initially identified as an odorous constituent of several  
74 marc distillates from Muscat cultivar (marc distillate of White Muscat from Piedmont,  
75 Catalan roxo cultivar marc distillate) and spirits (Cognac and Calva distillate) (Di Stefano,  
76 1986, Versini et al., 1995; Ledauphin et al., 2004). It was also tentatively identified and  
77 assayed in wines elaborated from grapes undergoing carbonic maceration (Dell'Oro & Di  
78 Stefano, 1990), where its quantitative levels depended on the type of grape variety. It was  
79 later assayed in white wines from *Vitis vinifera* Emir variety (Cabaroglu et al. 1997). It was  
80 mentioned as a wine component based on various analytical approaches, including  
81 Comprehensive GC (GCxGC) analysis (Robinson et al., 2011; Bordiga et al., 2014; Versini et  
82 al. 2005; Carlin et al. 2019a). It was also analysed at higher concentrations in experimental  
83 Cabernet Sauvignon wines elaborated with high proportions of petiole at harvest (Ward et al.  
84 2015) and in some white Italian grape varieties (Verdicchio and Trebbiano di Lugana) (Carlin  
85 et al. 2019a). In grapes and wine, MeSA was also observed in several varieties under a  
86 glycosylated form, liable to be released by chemical and enzymatic means (Williams et al.  
87 1989; Versini et al. 2005; Esti & Tamborra 2006; Ghaste et al. 2015; Carlin, 2019ab).

88 Based on previous research, the present study aimed to better characterize methyl  
89 salicylate in wines, study its range of concentrations, its sensory impact on wine aromas, its  
90 content during vinification, particularly in relation to the development of fungal diseases on  
91 *Vitis vinifera* grapevines (i.e., downy mildew, black rot, and Esca, a Grapevine Trunk  
92 Disease).

93

## 94 **2. Materials and Methods**

### 95 *2.1. Chemicals*

96 Dichloromethane (99.9%) was supplied by VWR Chemicals (Fontenay-sous-Bois,  
97 France). Sodium chloride (NaCl) was supplied by Supelco (Bellefonte, PA, USA). Ultrapure  
98 water was obtained from a Milli-Q Plus water system (Millipore, Saint-Quentin-en-Yvelines,  
99 France). Volatiles chemicals [methyl salicylate (analytical purity,  $\geq 99\%$ ), ethyl salicylate  
100 (99%), 3-octanol ( $\geq 99\%$ ), 1,8-cineole ( $\geq 99\%$ )], alkanes (C8–C20) in solution and  
101 compounds for fermentative media as cited by Marullo et al. (2006) were provided, all in  
102 reagent or ACS grade, by Sigma-Aldrich (Saint-Quentin-Fallavier, France). Volatiles stock  
103 blended solutions of 100 mg/L were prepared in HPLC-grade absolute ethanol (99.9%)  
104 (Merck, Fontenay-sous-Bois, France) and stored at + 4 °C.

105

### 106 *2.2. Wines samples*

107 French red wines (52 samples) from several origins (Bordeaux, Burgundy, Loire and  
108 Rhône Valleys, South West area) and vintages (from 2002 to 2014) were used in this study.  
109 Other samples (46) were from various Bordeaux vineyards (Table S1.).

110

### 111 *2.3. Grapes sampling*

#### 112 *2.3.1. Sampling of Merlot and Cabernet Sauvignon diseased and non-diseased grapes*

113 In 2012, a Cabernet-Sauvignon vine experimental plot from Bordeaux area was  
114 selected as previously mentioned (Pons et al., 2018). In the plot, 40 vines received fungicide  
115 treatment (10 times to control the main diseases as downy mildew, powdery mildew and  
116 black rot) while 40 vines didn't at any time during the growing season. At optimum ripening

117 stage, the grape bunches from each modality of the plot were harvested by hand, removing  
118 possible foci of grey rot (*B. cinerea*).

119 In 2013 and 2014, 40 kg of Merlot grapes were harvested on 6 blocks of a Bordeaux  
120 vineyard plot planted in 2011 at ISVV (Institut des Sciences de la Vigne et du Vin, Bordeaux)  
121 within the *ResIntBio* experimental vineyard (low-input viticultural cropping system). The  
122 visual estimates made it possible to place grape damage level by downy mildew (brown rot)  
123 due to *Plasmopara viticola*. This damage was situated between 2.2 and 59.3% depending on  
124 the block. In the same time, 40 kg of grapes from a downy mildew-resistance variety, *Artaban*  
125 (reference IJ134) developed in the context of an INRAE project, were harvested on 3  
126 vineyard blocks grown in the same place. These grapes were used for experimental  
127 vinifications.

128 In 2014, on the same Merlot ISVV vine plot, various categories of grape berries were  
129 harvested on the same day: healthy berries, naturally shrivelled berries due to sun exposure,  
130 berries shrivelled under the action of fungal disease such as downy mildew due to *P. viticola*  
131 and grape black rot due to *Guignardia bidwellii*.

132 In 2015, healthy Cabernet Sauvignon grapes were sampled in the control vines (20  
133 grape bunches harvested) and on the vines with strong foliar symptoms of grapevine trunk  
134 disease (Esca) (20 grape bunches) at the optimal ripeness stage in the same Haut-Médoc,  
135 Denomination of Appellation Origin vineyard plot (Bordeaux vineyard). In the laboratory,  
136 from these selected grapes, 100 berries without pedicels were randomly sampled from these  
137 grapes, frozen in liquid nitrogen, and introduced 2 min into a mechanical ball mill to be  
138 ground into a fine powder. Grape stems were analysed according to the same protocol of  
139 preparation. Each modality was performed in triplicate (n=3).

140 In 2016, 10 kg Cabernet Sauvignon grape bunches were harvested at optimal ripeness  
141 stage in Côtes-de-Bordeaux, Denomination of Appellation Origin, (Bordeaux vineyard) from

142 control vines, vine plants affected by grapevine trunk disease (Esca) with moderate leaf  
143 symptoms (level 1), and with strong leaf symptoms (level 2).

#### 144 2.3.2. *Sampling of Furmint grapes from diseased grapevine or not*

145 In 2017, *Vitis vinifera* Furmint B grapes were sampled at two harvest periods in Tokaj  
146 region (Hungary). The first harvest of healthy grapes, without infection of *B. cinerea*, was  
147 done at optimal ripeness stage for the elaboration of dry white wine. Two categories of  
148 harvest were considered, one on apparently healthy vine plants and the other on vine plants  
149 affected by strong leaf symptoms of grapevine trunk disease (Esca). The second harvest was  
150 done 1 month and half later (for elaboration of sweet wine) with berries affected by noble rot  
151 due to *B. cinerea* considering the same categories of grapes. For each harvest, a percentage of  
152 Esca diseased grape bunches were used to elaborate the wine in comparison with the control  
153 (from healthy vines).

154

#### 155 2.4. *Experimental winemaking conditions*

##### 156 2.4.1. *Red winemaking modalities*

157 In 2012, red wines were elaborated with Cabernet-Sauvignon grapes naturally infected  
158 by *P. viticola* (brown rot) and increasingly supplemented to control grapes (grapes from the  
159 fungicide treatment modality without brown rot) (Pons et al., 2018). During the experiments,  
160 incorporation was carried out in % of withered berries per cluster for the 5 modalities (0, 2, 5,  
161 10, 20 %). Each modality contained 60 kg of grapes vinified in 100 L stainless steel tanks  
162 after grapes destemming and crushing. Standard winemaking procedures were followed  
163 including alcoholic and malolactic fermentation with commercial yeast and bacterial strains  
164 (Laffort Œnologie, Floirac, France). At the end of malolactic fermentation, red wines were  
165 sulfited at 50 mg/L (6% v/v; Laffort, Bordeaux, France). Before bottling, they were fined and  
166 filtered. Then, wines were filled into 750 mL glass Bordeaux bottles and closed using



167 standard commercial practices. Sulfur dioxide was adjusted at 30 mg/L before bottling.  
168 Bottles were kept in a dark and in a temperature controlled room (18 °C) until required for  
169 analysis.

170 For vintages 2013, 2014, 2016, red wines were vinified at ISVV facility. All hand-  
171 harvested Merlot, Cabernet, Artaban grape bunches (20 kg each modality) were destemmed  
172 mechanically (mechanical destemmer, Bellot, Gradignan, France), manually crushed and  
173 vinified in stainless-steel tanks. Grape juices were inoculated with a commercial strain of  
174 *Saccharomyces cerevisiae* (FX10; Biolaffort Œnologie, Bordeaux, France) at 20 g/hL. In  
175 order to reproduce the winemaking conditions prevalent in the Bordeaux region, maceration  
176 lasted three weeks. During alcoholic fermentation (around 7 days), the cap was punched down  
177 twice per day. After spontaneous malolactic fermentation in 3 L bottles maintained at 20°C,  
178 wine were supplemented with 50 mg/L sulfur dioxide solution (6% v/v; Laffort, Bordeaux,  
179 France), bottled and kept at 18°C until analysis.

#### 180 2.4.2 White winemaking modalities

181 In 2017, dry and sweet Furmint white wines were vinified in Tokaj experimental  
182 winery. Vinification modalities were organized at each harvesting stage with incorporation of  
183 25%, 50% or even 75% of Furmint grape bunches from strongly Esca diseased vines. In each  
184 situation, grapes were crushed and the juices were put in 5L bottles per modality with 3  
185 replicates. Then, grape juices were inoculated with commercial *Saccharomyces cerevisiae*  
186 yeast strains *Uniferm 228* (Uniferm, Werne, Germany) for dry wine and *Actiflore B0213*  
187 (Laffort Œnologie, Bordeaux, France) for sweet wine at 20 g/hL. The dry white wines were  
188 sulfited at 70 mg/L at the end of alcoholic fermentation (less than 2 g/L residual sugars) then  
189 kept in bottles at 18°C until analysis. The sweet wines were sulfited at 140 mg/L when  
190 alcohol strength reached 12% vol. then kept in bottles until analysis.

191

192 *2.5. Laboratory scale microvinifications with incorporation of diseased berries*

193 With Merlot grape berries from 2014 vintage (healthy, naturally shrivelled berries due  
194 to sun exposure or shrivelled under the action downy mildew and grape black rot), 20 selected  
195 berries of each type were fermented in a 100 mL-bottle containing 60 mL of a model must  
196 solution with a composition described by Marullo et al., 2006. After alcoholic fermentation,  
197 lees were removed by centrifugation (5 min, 6000 r.p.m.), samples were sulfited (50 mg/L)  
198 and stored at 4 °C before analysis experiments. Each modality was performed in triplicate  
199 (n=3).

200

201 *2.6. Sample preparation for Methyl salicylate (MeSA) evidence in wine*

202 *2.6.1. Wine extraction and semi-preparative HPLC*

203 As described by Poitou et al. (2017), a 750 mL Cabernet wine sample was extracted  
204 using 80, 50, and 50 mL dichloromethane for 10 min each with magnetic stirring (700 r.p.m.)  
205 and separated in a funnel. The organic phases were collected, frozen overnight at -20°C to  
206 remove the emulsion, and then dried over sodium sulfate and concentrated to around 20 mL  
207 using a Buchi R-114 rotary evaporator (Buchi, Rungis, France). The extract was filtered using  
208 a 0.45 µm membrane syringe and concentrated under a nitrogen flow (100 mL/min) in a  
209 graduated glass tube (Jean Premont, Bordeaux) to obtain 500 µL wine extract. Then, the  
210 extract was injected on to Reverse-phase (RP) HPLC system using water and ethanol as  
211 solvents. the Ultimate 3000 semipreparative HPLC system (Dionex, Courtaboeuf, France) and  
212 a Novapak C18 column (300 × 7.8 mm internal diameter (i.d.), 6 µm, Waters, Saint Quentin,  
213 France) with a guard column of the same phase.

214 Chromatographic conditions included a 250 µL injection volume with a flow rate of 1  
215 mL/min. The linear program gradient was as follows: phase A, water; phase B, ethanol; 0–2

216 min, 100 % A, 0–50 min, linearly programmed until 100 %. Fifty fractions, containing each 1  
217 mL effluent, were collected and evaluated for their sensory properties.

218 Selected fractions were mixed with the next or previous fraction on the basis of their  
219 odour to form a 2 mL fraction group. Each was then diluted with ultrapure water to obtain 12  
220 % ethanol (v/v) and then re-extracted three times with 10 % (v/v), 5 % (v/v), and 5 % (v/v)  
221 dichloromethane, respectively, for 10 min each time. The organic phases were combined and  
222 concentrated to 100 µL under nitrogen flow before analysis.

223

#### 224 *2.6.2. Identification of MeSA by Heart-cut Gas Chromatography coupled to Olfactometry and* 225 *Mass Spectrometry (MDGC-O-MS)*

226 Heart-Cut Multidimensional gas chromatography separation was performed on two  
227 Agilent 7890 GC (Agilent Technologies, Palo Alto, CA, USA), connected via a heated  
228 transfer line at 230°C as previously mentioned (Poitou et al. 2017).

229

#### 230 *2.7. Wine sample treatments for studying MeSA release from bound forms*

231 Red wine samples were acidified at pH 3.0 with addition of 5M H<sub>2</sub>SO<sub>4</sub> and aliquoted  
232 in 20-mL headspace amber vials before closure with a PTFE-faced silicone septum/aluminum  
233 crimp cap. They were then kept in the dark at 60°C prior to analysis or at 20°C for control.

234

#### 235 *2.8. Quantitation of MeSA, ethyl salicylate (EtSA) and 1,8-cineole.*

236 Concentrations of MeSA, EtSA and 1,8-cineole were determined by solid-phase  
237 microextraction coupled to gas chromatography–mass spectrometry (SPME-GC-MS). MeSA  
238 concentrations were also determined in comparison, by solid-phase-extraction coupled to gas  
239 chromatography–mass spectrometry (SPE-GC-MS) analysis on selected samples. For each  
240 modality, analyses were performed using a Combi PAL sampler (CTC Analytics, Zwingen,

241 Switzerland) on an Agilent 6890N gas chromatograph (Agilent, Palo Alto, CA, USA),  
242 coupled to an Agilent HP 5973N mass spectrometer (electron impact mode at 70 eV).

243

#### 244 *2.8.1. Quantitation of MeSA, EtSA and 1,8-cineole by SPME procedure*

245 A 7 mL sample diluted in deionized water containing 5 mL grape juice, wine or 3 g  
246 grape-berry powder was transferred into a 20-mL headspace amber vial containing 3 g  
247 sodium chloride (NaCl). A 10  $\mu$ L sample of internal standard solution 3-octanol at 100 mg/L  
248 in ethanol was supplemented before closure with a PTFE-faced silicone septum/aluminum  
249 crimp cap and homogenized manually.

250 A 2 cm, 50/30  $\mu$ m Divinylbenzene-Carboxen-Polydimethylsiloxane  
251 (DVB/CAR/PDMS) SPME fiber purchased from Supelco Inc. (Bellefonte, PA, USA) was  
252 used. The sample was pre-incubated for 5 min at 40°C. Adsorption lasted 30 min, at the same  
253 temperature, with stirring at 500 r.p.m. (3 seconds on, 2 seconds off). Then, desorption took  
254 place in the injector in splitless mode (3 min) at 240 °C for a duration of 10 min, with a purge  
255 flow rate of 50 mL/min. The fiber was then reconditioned for 10 min at 250 °C.

256

#### 257 *2.8.2. Quantitation of MeSA by SPE procedure*

258 The isolation of MeSA from wine was done by its percolation on cartridge sorbents  
259 using an automatic liquid handler GX-274 ASPEC (Gilson, Middleton, WI). Initially, the  
260 cartridge sorbents [SPE Chromabond HRX, 500 mg sorbent, 6 mL cartridge volume from  
261 Macherey-Nagel (Bethlehem, PA, USA)] were conditioned with 7 mL of methanol at a 6  
262 mL/min flow, followed by a 3 mL volume of ultrapure water/ethanol (90:10, v/v) mix at the  
263 same flow. Then, to a 20 mL of wine sample were supplemented 50 $\mu$ L of 3-octanol at 100  
264 mg/L in ethanol. 19640  $\mu$ L of this mixed wine sample were percolated on the cartridge at a  
265 flow of 3 mL/min. After loading, the cartridge was washed with 2 mL of ultrapure water, then

266 compounds were eluted with 3 mL of dichloromethane/pentane (50:50, v/v) and 3 mL of  
267 dichloromethane/methanol (95:5, v/v). Percolation speed was fixed at 10 mL/min. The  
268 organic phases were combined, dried with sodium sulfate, concentrated under nitrogen flow  
269 (at approximately 100 mL/min) to 150  $\mu$ L and kept  $-20$   $^{\circ}$ C before analysis.

270

### 271 2.8.3. GC-MS analysis.

272 The carrier gas was Helium N60 (Air Liquide) with a flow rate of 1 mL/min. A  
273 Carbowax 20 M type fused capillary column was used: BP20, 50 m, 0.25 mm i.d., 0.22  $\mu$ m  
274 film thickness (SGE, Ringwood, Australia). Temperature program was as follows: 45  $^{\circ}$ C for 5  
275 min, increasing temperature by 3  $^{\circ}$ C/min to 180  $^{\circ}$ C then 20  $^{\circ}$ C/min to 240  $^{\circ}$ C with an  
276 isotherm at the final temperature for 10 min. The mass spectrometer, operating in electron  
277 impact (EI) mode (70 eV), was connected to the GC with a heated transfer line at 230  $^{\circ}$ C. The  
278 compounds were quantitated using selected ion monitoring mode (SIM) on MSD Chemstation  
279 software (v B.04.03) from Agilent. Selected ions for internal standards were  $m/z$  83, 59 (3-  
280 octanol), and  $m/z$  83 for quantitation. MeSA was detected using  $m/z$  152, 120, 92 ions, and  
281 quantitated using  $m/z$  152 ion. EtSA was detected using  $m/z$  166, 120, 92 ions, and quantitated  
282 using  $m/z$  120 ion. 1,8-cineole was detected using  $m/z$  154, 139, 111, 108 ions, and  
283 quantitated using  $m/z$  108 ion.

284

### 285 2.8.4. Method validation for MeSA, EtSA and 1,8-cineole quantitative analysis

#### 286 2.8.4.1. SPME-GC-MS analysis

287 **Linearity** (n=3), estimated by a spike at 6 increasing concentration levels of MeSA  
288 and EtSA (5/10/25/50/100/200  $\mu$ g/L), were 0.998 and 0.993 respectively. **Precision** (n=5)  
289 estimated as the relative standard deviation (% RSD) were 2.87 % and 5.84% respectively.  
290 The **recovery** values (% RSD) estimated by adding 30  $\mu$ g/L to a reference wine were 104.5 %

291 and 98.7 % respectively. **Limit of Quantitation (LOQ, S/N 10)** were estimated at 1.05 µg/L  
292 and 0.41 µg/L for MeSA and EtSA respectively. **Limit of Detection (LOD, S/N 3)** were  
293 estimated at 0.35 µg/L and 0.13 µg/L for MeSA and EtSA respectively. Method validation for  
294 1,8-cineole quantitative analysis was described elsewhere (Poitou et al. 2017).

295 Validation for berry analysis was performed in deionized water following the same  
296 procedure. Values were equivalent for the various parameters calculated. Results were  
297 reported on a per weight basis in micrograms per kilogram of fresh weight for berries and per  
298 litre for wines.

#### 299 2.8.4.2. SPE-GC-MS analysis

300 **Linearity** (n=3) estimated by a spike at 5 increasing concentration levels of MeSA  
301 (1,25/2,5/5/10/20 µg/L) was 0.997. **Precision** (n=3) estimated as the relative standard  
302 deviation (% RSD) was 3.44 %. **LOQ** and **LOD** were estimated respectively at 0.03 µg/L and  
303 0.01 µg/L.

#### 304 2.8.4.3. Comparative accuracy of methyl salicylate assay by SPE-GC-MS and SPME-GC-MS

305 The determination of methyl salicylate in wines by the SPE GC-MS method and by the SPME  
306 GC-MS method were compared on the accuracy parameter using the protocol defined in the  
307 Compendium of international methods of wine and must analysis (2020), which refers to ISO  
308 13528 (2015).

309

### 310 2.9. Sensory Analysis

#### 311 2.9.1. General conditions.

312 Sensory analyses were conducted by 2 panels from the research unit in Enology  
313 (enologists, researchers, or students) with a good experience of wine tasting (usual tasting  
314 wine several times a week). The panelists (n = 27 for first session, n=18 for second and third  
315 sessions) were aged between 22 and 56 years old. 77% and 61% respectively, depending on

316 the session, were having less than 35 years old (average age 29 years old for the first session,  
317 and 33 years old for the second and third sessions). The percentage of female panelists was 67  
318 % and 72 % respectively. Orthonasal sensory evaluations took place in a temperature-  
319 controlled room (ISO 8589:2007) maintained at  $20 \pm 1^\circ\text{C}$ , equipped with individual boxes.  
320 Samples were presented in random order, in black tasting glasses, coded by three-digit  
321 numbers, and covered with plastic caps. All samples were exclusively smelled by orthonasal  
322 evaluation. Generally, sensory evaluations were conducted during a unique session of 3 h  
323 with a renewal of the samples at mid-session. In the context of the COVID-19 epidemic  
324 (second and third sessions), the olfactory detection thresholds were conducted all a day, by  
325 changing the glasses and refilling them, after each experimenter had reached twice the  
326 olfactory detection threshold, with a time volume of 15 minutes between each. Between each  
327 repetition, the order of the glasses and the coding were changed.

328

### 329 *2.9.2. Determination of MeSA olfactory detection threshold.*

330 Olfactory detection threshold was determined in model wine [L(+)-tartaric acid 5 g/L,  
331 12 % vol., pH 3.5]. Samples were presented as a series of triangular tests with ascending  
332 order of methyl salicylate concentration: 11.8, 17.7, 26.6, 40, 60, 90, 135, 202.5  $\mu\text{g/L}$   
333 respectively. Value was calculated using the BET (Best Estimate Threshold) method (NF ISO  
334 13301: 2002). For each panelist, the best estimate threshold was the geometric mean of the  
335 highest concentration missed and the next higher concentration tested. BET for the group is  
336 the result of the geometric mean of the individual BETs. The odor active value (OAV) which  
337 represents the measured concentration of a volatile present in a sample, divided by its  
338 measured sensory detection threshold in a similar matrix, was also determined on selected  
339 wine samples (Patton and Josephson, 1957).

340

341 2.9.3. *Evaluation by a free vocabulary task.*

342 26 panelists from the first panel (17 women, 9 men; average age 28 years old)  
343 expressed their olfactory perception with their own terms during a single session. A unique  
344 modality containing 200 µg/L of methyl salicylate, was evaluated in a model wine [L(+)-  
345 tartaric acid 5 g/L, 12 % vol., pH 3.5] and in a non-barrel-aged, commercial Bordeaux wine.  
346 Terms with only one citation or non-adapted (hedonic, specific of taste) were not considered.  
347 Several were also considered together on the basis of their analogy.

348

349 2.10 *Statistical analysis.*

350 Analysis of variance (ANOVA) was performed on normalized scores (judges),  
351 homogeneity of variance was evaluated with Levene's test, and the normality of residuals was  
352 assessed using the Shapiro–Wilk test. Statistical significance was set at 5 % ( $p < 0.05$ ).  
353 Statistics were calculated using R software (v 3.1.1) except Spearman correlation test with  
354 XLStat-Premium software (Addinsoft).

355

### 356 **3. Results and discussion**

357 3.1. *Methyl salicylate as an odorous volatile compound in experimental and commercial red*  
358 *wines*

359 In a past publication, while performing GC-O analysis of an HPLC fraction from an  
360 extract of an unripe Bordeaux Cabernet-Sauvignon experimental wine, several odorous zones  
361 were detected that corresponded to several impact volatile compounds, such as 2-methoxy-3-  
362 alkylpyrazines, 1,8-cineole, 1-(2,3,6-trimethylphenyl)buta-1,3-diene, and several C6, C8, C9  
363 saturated and unsaturated aldehydes (Poitou et al. 2017). When this approach was pursued  
364 using other extracts, an odorous zone marked by fresh, camphor and medicinal notes was  
365 detected, with linear retention indices of 1775 and 1202 on BP20 and BP1 respectively,



366 corresponded to the retention time of methyl salicylate (MeSA). Identification was confirmed  
367 by mass spectrum by comparison to NIST library and by injection of the pure commercial  
368 standard.

369 MeSA was then quantified by SPME-GC-MS method, in a series of 98 French red  
370 wines, including 52 commercial wines from different wine-producing regions (Bordeaux,  
371 Loire and Rhône valleys, Burgundy, etc.). The remaining wines were experimental wines  
372 made from various clones of Cabernet Franc and Merlot grapes. The numerical quantitative  
373 data was graphically depicted by a boxplot (**Fig. 1**).

374 The quantitative results showed that the methyl salicylate concentrations in the  
375 analysed wines mainly ranged between 5 and 25  $\mu\text{g/L}$ , with about 15% of the wines ranging  
376 between 30 and 40  $\mu\text{g/L}$ . The mean and the median of the 98 wines were 18.7  $\mu\text{g/L}$  and 13.6  
377  $\mu\text{g/L}$ , respectively, which is similar to the values observed by Carlin et al. (2019a) in  
378 Verdicchio wines. However, proportionally high levels were observed in some wines ( $n = 5$ ),  
379 with concentrations ranging from 69.2 to 131.8  $\mu\text{g/L}$  (**Table 1**). These 5 wines corresponded  
380 to 3 commercial wines from the 2014 vintage (Côtes-du-Rhône, Irouleguy and Bordeaux  
381 Denominations of Appellation Origin) and two experimental Merlot wines obtained from  
382 different soils (gravel and sand) from the 2006 vintage. The accuracy of the MeSA  
383 quantitative measurements led by SPME-GC-MS was confirmed by assays done on selected  
384 wine samples using an alternative method by SPE-GC-MS (**Table S2**).

385 Analysis of ethyl salicylate (EtSA), the structural analogue with a similar odour,  
386 revealed its presence in these wines, but at much lower concentration levels, ranging from 0.3  
387 to 9.8  $\mu\text{g/L}$  with a mean of 0.91  $\mu\text{g/L}$ . Furthermore, 1,8-cineole, another odorous marker of  
388 green eucalyptus notes in red wines, presented concentrations that did not exceed 2.24  $\mu\text{g/L}$ ,  
389 which were not related to those of MeSA and EtSA (**Table 1**). Concerning the assay of MeSA  
390 in wines from Cabernet Franc clones grown on the same plot, the results showed variability

391 between clones from the same vintage (10-40%), and even greater variability in different  
392 vintages, with variations in average concentrations ranging from 1.5 to 4.8 fold (**Fig. S1**).

393

394 *3.2. Sensory impact of methyl salicylate in red wines evaluated through psychophysical tests*  
395 *and sensory reconstitution*

396 The olfactory detection threshold of methyl salicylate was determined three times in a  
397 model wine solution. Value estimation (BET method) revealed an olfactory detection  
398 threshold of  $75 \pm 25 \mu\text{g/L}$  in the model wine solution. For each olfactory session, the  
399 minimum detection threshold value by certain panel members was  $9.6 \mu\text{g/L}$ . These values  
400 indicated that MeSA could have a sensory contribution at the highest concentration levels  
401 assayed in red wines (**Table 1**). Synergistic effects were observed between MeSA and  
402 guaiacol, an odorous oak wood volatile compound (Yodder et al. 2012). Furthermore, MeSA  
403 could be involved in perceptual interaction phenomena with other wine volatile compounds.

404 In order to qualitatively characterize the odour change caused by an increase of MeSA  
405 concentration in the wine, a panel of 26 wine experts conducted analysis using a free  
406 vocabulary technique. An addition of  $200 \mu\text{g/L}$  of this molecule to red wine introduced an  
407 aromatic freshness expressed by an increase in the number of citations of the terms “fresh” or  
408 “fresh fruit”. Specific fresh green nuances were also noted in the model wine as “chemical”,  
409 “dentist”, “pharmaceutical”, “menthol”, and “camphor” (**Table S3**).

410

411 *3.3. Location of MeSA in healthy grapes (berries, stem), must and extraction kinetics during*  
412 *red wine vinification.*

413 To determine the distribution of methyl salicylate in the different parts of the grape,  
414 quantitation was performed after separating the berries and stems and on the pulp and skins.  
415 Analysis carried out on Cabernet Sauvignon grapes harvested on healthy plants at optimal

416 ripeness (2015 vintage) revealed that concentrations of MeSA in stems (122  $\mu\text{g}/\text{kg}$  fresh  
417 weight) were significantly higher than concentrations in berries (0.5  $\mu\text{g}/\text{kg}$  fresh weight).  
418 These observations were consistent with those of Ward et al. (2015), which showed an  
419 increase in methyl and ethyl salicylate concentrations, as well as several terpenes, with an  
420 incorporation of petioles in the must. Further analysis revealed that methyl salicylate was  
421 more abundant in the skins (68%) than in the pulp (32%). After harvesting, quantitation of  
422 MeSA was performed on must from healthy Cabernet Sauvignon cultivar grapes. MeSA was  
423 progressively extracted during alcoholic fermentation and post-fermentation maceration. The  
424 assay of MeSA in the must therefore showed a gradual increase in its concentrations during  
425 vinification (**Fig. 2.**).

426 The maximum concentration was reached at the end of fermentation, after approximately ten  
427 days. Furthermore, since glycosylated forms of MeSA had already been observed in grapes  
428 and wine (Williams et al. 1989; Esti et al. 2006; Ghaste et al. 2015; Carlin et al. 2019b), the  
429 increase in MeSA observed during alcoholic fermentation may result from a combined effect  
430 of extraction (skin) and release through the enzymatic action of yeast, as observed with  
431 monoterpene glycosides (Bisotto et al., 2015). Moreover, an acidic treatment, at pH 3,  
432 performed on several red wine samples stored at 20°C (control) and at 60°C led to increased  
433 MeSA concentrations in the wines (**Table S4.**). This observation supports the existence of  
434 “bound” forms of this compound in these wines, presumably related to glycosides, as  
435 previously mentioned.

436 Moreover, since MeSA levels appear to be much higher in the stems, red wine  
437 vinification procedures, with and without destemming, were performed according to  
438 Bordeaux red winemaking protocol, with 100% destemmed grapes, or the incorporation of  
439 non-destemmed grapes (20% in volume). The results showed an increase in MeSA levels in

440 wines (82% of increase in free run wines) elaborated with non-destemmed grapes (**Table**  
441 **S5.**).

442

443 *3.4. Detection and quantitation of methyl salicylate in grapes, musts and wines from diseased*  
444 *vines infected with cryptogamic fungi*

445 Considering the possible origins of MeSA, as stated in the introduction, the hypothesis  
446 of a defence reaction to infection by various grapevine pathogens (downy mildew; grapevine  
447 trunk disease, ESCA) was naturally considered to interpret the high content measured in  
448 several wines.

449 *3.4.1. Impact of downy mildew and other grape pathogens*

450 Two series of wines were considered for MeSA analysis. The first series was produced  
451 from Merlot grapes affected by downy mildew (*P. viticola*), with estimated proportion  
452 ranging from 2.2 to 59.3% from grape bunches with visual damage (10 wines). The second  
453 series was produced from the newly created *Vitis* sp. variety *Artaban*, which is resistant to  
454 downy mildew (4 wines). All of the grapes had been vinified under the same conditions.  
455 MeSA content was assayed in these experimental wines in 2 vintages (2013-2014). MeSA  
456 was therefore assayed in Merlot wines from grapes infected with downy mildew with  
457 concentrations of 120.7 µg/L, and a maximum value of  $157.9 \pm 6.7$  µg/L, while  
458 concentrations were below 29.8 µg/L in wines from resistant varieties, without any downy  
459 mildew symptoms (**Fig. S2.**).

460 The concentration of MeSA was the assayed in experimental Cabernet-Sauvignon  
461 wines from 2012 vintage, elaborated with the incorporation of various proportions of grapes  
462 infected by downy mildew. This analysis had previously been performed by Pons et al. (2018)  
463 for other volatile compounds (lactones, 2-methoxy-3-isobutylpyrazine, 3-methyl-2,4-  
464 nonanedione) on samples kept in a wine cellar. In this case, while no significant differences

465 were observed in the usual analysis between the modalities, a proportional increase of MeSA  
466 content was observed when berries infected with brown rot were incorporated (**Table 2,**  
467 **Table S6.**). The results showed MeSA content ranging from 8.46 µg/L in wines obtained  
468 from must without any addition of diseased berries to 32.14 µg/L in wines obtained with an  
469 incorporation of 20% diseased berries.

470 In addition, the impact of other grapevine pathogens on MeSA content in wines was  
471 considered through fermentation in model must supplemented with selected berries of each  
472 type: healthy, shrivelled by the sun, shrivelled by the effects of downy mildew (*Plasmopara*  
473 *viticola*) and infected grape black rot (*Guignardia bidwellii*). The same number of Merlot  
474 grape berries (20 berries), harvested in 2014, were incorporated into the model must and  
475 fermented. It is important to note that, unlike berries affected by downy mildew, the solutions  
476 obtained after the alcoholic fermentation and maceration of berries infected with grape black  
477 rot were colourless. Analysis of the solutions revealed a slight increase in the concentrations  
478 of MeSA linked to the shrivelling phenomenon in the berries. However, variations between  
479 the samples remained low, particularly in comparison to those generated by the two grapevine  
480 pathogens, mildew and grape black rot (**Fig. 3.**).

481 Therefore, all the experiments confirmed the relationship between MeSA content in  
482 wine and downy mildew, as suggested in the vine *V. vinifera* (Chalal et al., 2015). Moreover,  
483 it was demonstrated that other fungi, such as grape black rot, can also induce significantly  
484 higher concentrations of this compound in berries and in wine. Furthermore, we observed that  
485 fermentation of the model must solution with 1 mg/L of salicylic acid did not induce any  
486 distinct increase in the concentrations of methyl salicylate (results not reported). This shows  
487 that it cannot have originated from the metabolism of salicylic acid by yeast, which is  
488 possibly present in greater quantities in the infected plants and grapes.

489

490 3.4.2. *Esca*, grapevine trunk disease impact

491 The impact of *Esca* (one Grapevine Trunk Disease) on methyl salicylate  
492 concentrations in wines was also considered. For this purpose, Cabernet Sauvignon grapes  
493 from healthy plants and *Esca*-affected plants with moderate or strong leaf symptoms were  
494 harvested (2016 vintage) and vinified under the same conditions. Classical wine analysis  
495 evidenced a slightly lower alcoholic strength of wines from *Esca*-affected vines, in  
496 accordance with previous studies (Lorrain et al., 2012) (**Table S7**). The quantitation of  
497 MeSA was carried out on samples collected immediately after alcoholic fermentation was  
498 complete, then one week later in the wines in contact with the grape pomace, and a year later  
499 for the final wines. Quantitation of MeSA showed a significant increase of its concentrations  
500 in wines linked to the *Esca* infected vine plants (**Table 3**). Moderate damage as expressed by  
501 leaf symptoms was sufficient to double the MeSA concentrations compared to the control.  
502 Severe *Esca* symptoms in the plant led to an even greater increase in concentrations compared  
503 to the control. Quantitative results one year later in the aged wine confirmed the results  
504 obtained immediately following the end of alcoholic fermentation. This experimentation  
505 therefore confirmed the real influence of *Esca* grapevine disease on the increase of MeSA  
506 content in red wines. However, under these conditions, the concentrations remained low  
507 compared to the previously determined olfactory detection threshold of the compound.  
508 Indeed, concentrations of MeSA in wines are also dependent on other factors such as those  
509 stated above, and perhaps on the vine's ability to synthesize it. On the other hand, a  
510 comparative analysis of MeSA content in berries and stems from Cabernet Sauvignon grapes  
511 (Haut-Medoc, 2015), harvested from grapevines both severely affected and unaffected by  
512 *Esca*, showed increases in MeSA concentrations in stems from diseased grapes that were  
513 similar to those observed in the berries (**Table S8**).

514 Another study conducted an analysis of Tokaj white wines made with increasing  
515 proportions of grape from *Vitis vinifera* of the Furmint variety, harvested from Esca-infected  
516 vine plants (25%, 50%, 75 %) in comparison with the control. While the alcohol content was  
517 slightly lower in the wines containing a higher proportion of Esca, the quantities of MeSA  
518 were also higher in Esca wines than in the control dry white wine (**Table S9**). However, for  
519 the study carried out on the same vineyard with Furmint grapes affected by noble rot caused  
520 by *Botrytis cinerea* for the elaboration of sweet wines, the level of MeSA were similar in all  
521 modalities (**Table S9**).

522

### 523 **Conclusion**

524 Methyl salicylate (MeSA), as a volatile odorous compound, can be present in red wines at  
525 sometimes relatively high levels (70 to 130 µg/L), that is, concentrations near or above its  
526 olfactory detection threshold as determined in a model solution at  $76.2 \pm 25.5$  µg/L. Based on  
527 sensory experiences with supplementation at 200 µg/L, this compound could contribute to  
528 strengthening the expression of fresh green aromatic nuances in red wines, e.g.  
529 "pharmaceutical", "camphor" or "menthol" aromas. Furthermore, the significant concentration  
530 of MeSA in stems provides evidence that their supplementation during red wine vinification  
531 may increase the abundancy of this compound in wines. In addition, the relation between  
532 MeSA and several vine pathogens (downy mildew, grape black rot) or grapevine trunk  
533 disease (Esca) was demonstrated in grapes and in red and white wines. The vinification of  
534 such diseased grapes may affect the wine aroma quality, particularly when stems are  
535 incorporated. It appears that this change was induced by a host defence mechanism against  
536 fungal infection. Therefore, methyl salicylate may constitute a good volatile indicator of the  
537 vineyard's state of infection, revealing the physiological state of vine plants under fungal  
538 pressure.

539

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543

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547

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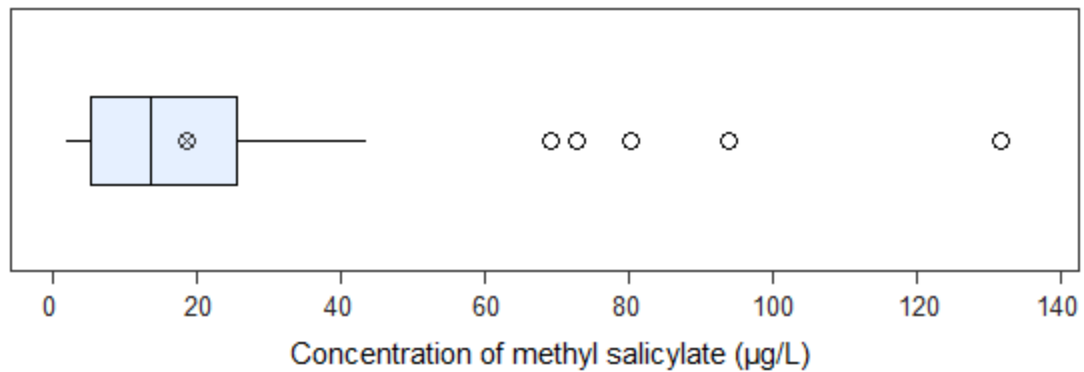
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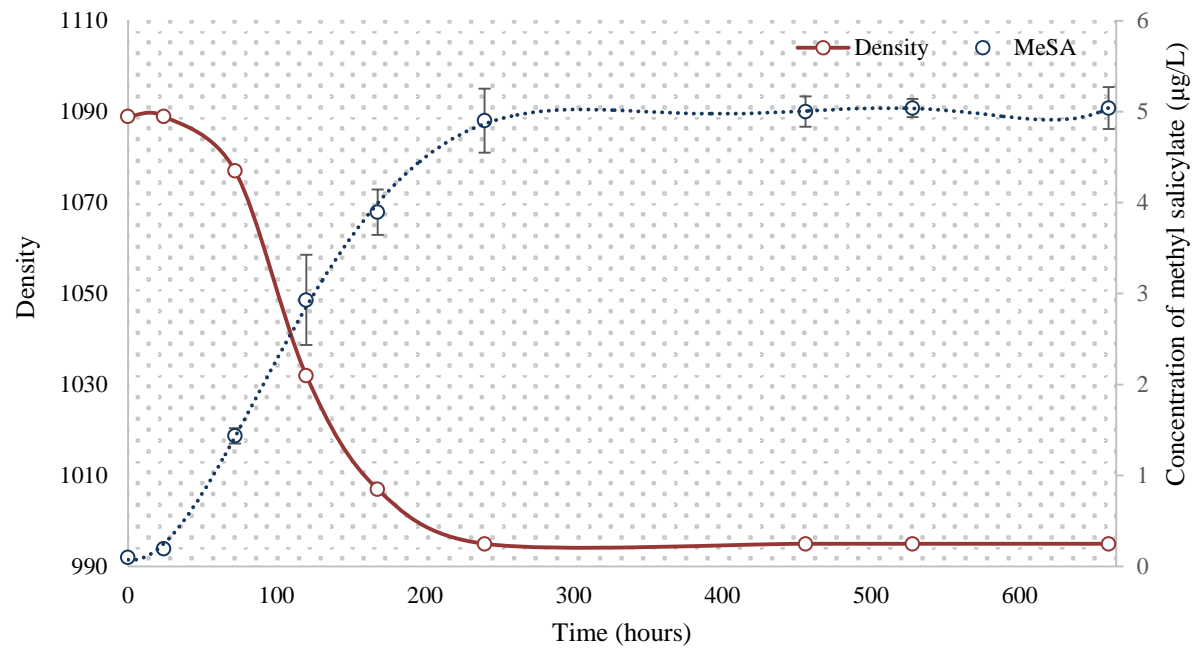
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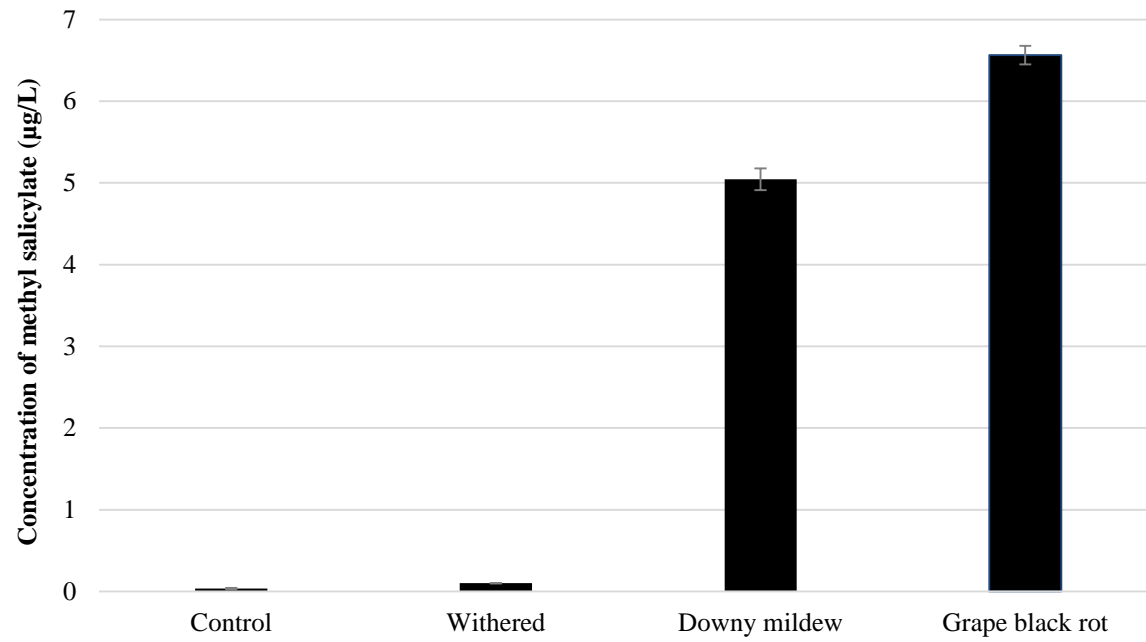
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**Table 1.** Methyl salicylate concentrations in various French red wines in comparison with ethyl salicylate and 1,8-cineole content (n=2)

		Volatile compounds <sup>a</sup>		
Vintage		Methyl salicylate (µg/L)	Ethyl salicylate (µg/L)	1,8-Cineole (µg/L)
Bordeaux (Merlot, on sand)	2006	<b>131.80 (19)</b>	0.79 (0.1)	0.07 (0.01)
Côtes du Rhône (Shiraz)	2014	<b>94.04 (0.5)</b>	2.82 (0.01)	0.2 (0.01)
Bordeaux (CS <sup>b</sup> , Merlot blend)	2014	<b>80.36 (6.0)</b>	7.61 (0.5)	0.16 (0.02)
Bordeaux (Merlot, on gravels)	2006	<b>72.94 (3.3)</b>	<b>0.74 (0.1)</b>	<b>0.05 (0.01)</b>
Bordeaux (hard press wine)	2014	<b>69.20 (1.0)</b>	<b>4.76 (0.1)</b>	<b>0.1 (0.01)</b>
Irouleguy (Cabernet franc)	2013	43.30 (0.1)	1.94 (0.1)	0.08 (0.01)
Bordeaux (Merlot, on clay)	2006	38.71 (1.5)	0.73 (0.2)	0.15 (0.01)
Chinon (Cabernet franc)	2014	38.62 (0.5)	1.06 (0.1)	0.17 (0.03)
Bordeaux (Merlot, on sand)	2007	37.54 (0.5)	1.01 (0.1)	0.21 (0.02)
Bergerac (Merlot, organic wine)	2013	37.15 (1.5)	0.29 (0.1)	0.23 (0.02)
Girondas (Grenache)	2012	35.24 (2.5)	1.36 (0.1)	0.39 (0.01)
Chateauneuf du Pape (Grenache)	2014	34.90 (4.0)	1.61 (0.1)	0.28 (0.01)
Fronton	2007	34.50 (0.5)	2.19 (0.1)	0.85 (0.01)
Bordeaux (Merlot, on clay)	2007	30.60 (0.5)	1.01 (0.1)	0.38 (0.01)
Valréas (Grenache, organic wine)	2014	30.36 (1.5)	0.98 (0.1)	0.26 (0.01)
Saint Estèphe (CS <sup>b</sup> , Merlot blend)	2014	26.32 (1.5)	5.37 (0.3)	0.13 (0.01)
Pauillac (CS <sup>b</sup> , Merlot blend)	2014	25.80 (4.0)	8.08 (2.0)	<b>1.04 (0.1)</b> <sup>c</sup>
Pauillac (CS, Merlot blend)	2009	25.67 (0.1)	9.81 (0.1)	<b>2.24 (0.03)</b>
Pauillac (CS, Merlot blend)	2013	23.29 (4.5)	0.63 (0.7)	0.29 (0.05)
Chinon (Cabernet franc)	2004	18.66 (0.6)	0.51 (0.1)	0.26 (0.04)
Margaux (CS, Merlot blend)	2004	17.32 (0.1)	1.10 (0.1)	0.31 (0.01)

<sup>a</sup> For each compound, concentrations in bold corresponded to OAV (Odor Activity Value) >1.

<sup>b</sup> CS : Cabernet Sauvignon <sup>c</sup> Poitou et al. (2017)

**Table 2.** Incidence of increasing content of diseased berries infected by *Plamospara viticola* on volatile compounds in Cabernet Sauvignon wines. <sup>a</sup> (n=2)

	diseased berries (%) <sup>b</sup>					RC <sup>c</sup>
	0	2	5	10	20	
Methyl salicylate (µg/L)	8.46 (0.9)	19.39 (1.4)	24.36 (0.05)	29.42 (2.5)	32.14 (1.65)	$p = 0.017$
Ethyl salicylate (µg/L)	1.42 (0.18)	1.36 (0.15)	1.13 (0.04)	1.39 (0.3)	1.11 (0.06)	n.s.
1,8-cineole (µg/L)	0.10 (0.005)	0.09 (0.005)	0.10 (0.01)	0.09 (0.01)	0.10 (0.01)	n.s.

<sup>a</sup> Analysis done 6 years after bottling.

<sup>b</sup> Results concerning wines made with healthy grapes and grapes infected with increasing % of berries infected by brown rot (*P. viticola*)

<sup>c</sup> RC : Regression coefficient with Spearman correlation test; n.s. = non significant

**Table 3.** Concentration of methyl salicylate in wines from healthy vines (control) or elaborated from grapes harvested on vine affected by grapevine trunk disease (Esca) (n=2)

Grape variety	Modality	Methyl salicylate ( $\mu\text{g/L}$ )
<b>Cabernet Sauvignon (red wine, 2016)</b>	Control	0.47 (0.25) <sup>a</sup> / 0.37 (0.1) <sup>b</sup>
	Esca level 1	1.25 (0.17) / 0.68 (0.05)
	Esca level 2	5.34 (0.93) / 1.48 (0.23)
<b>Furmint (dry white wine, 2017)</b>	Control	0.06 (0.02) <sup>b</sup>
	Incorporation level 1	0.16 (0.01)
	Incorporation level 2	0.42 (0.06)
	Incorporation level 3	0.62 (0.02)

<sup>a</sup> Concentration determined after 1 year ageing or <sup>b</sup> just after the end of alcoholic fermentation