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ABSTRACT.

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

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

1. Introduction

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

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

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

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

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

2. Materials and Methods

2.1. Chemicals

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

2.2. Wines samples

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

2.3. Grapes sampling

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

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

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

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

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

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

In 2016, 10 kg Cabernet Sauvignon grape bunches were harvested at optimal ripeness 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

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

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

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

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

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

Red wine samples were acidified at pH 3.0 with addition of 5M H_2SO_4 and aliquoted in 20-mL headspace amber vials before closure with a PTFE-faced silicone septum/aluminum crimp cap. They were then kept in the dark at 60°C prior to analysis or at 20°C for control.

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

Concentrations of MeSA, EtSA and 1,8-cineole were determined by solid-phase microextraction coupled to gas chromatography−mass spectrometry (SPME-GC-MS). MeSA concentrations were also determined in comparison, by solid-phase-extraction coupled to gas chromatography−mass spectrometry (SPE-GC-MS) analysis on selected samples. For each 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 µL 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 µm 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 50µL of 3-octanol at 100
264 mg/L in ethanol. 19640 µL 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

compounds were eluted with 3 mL of dichloromethane/pentane (50:50, v/v) and 3 mL of dichloromethane/methanol (95:5, v/v). Percolation speed was fixed at 10 mL/min. The organic phases were combined, dried with sodium sulfate, concentrated under nitrogen flow (at approximately 100 mL/min) to 150 µL and kept −20 °C before analysis.

2.8.3. GC-MS analysis.

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

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

2.8.4.1. SPME-GC-MS analysis

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

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

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

2.8.4.2. SPE-GC-MS analysis

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

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

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

2.9. Sensory Analysis

2.9.1. General conditions.

Sensory analyses were conducted by 2 panels from the research unit in Enology (enologists, researchers, or students) with a good experience of wine tasting (usual tasting wine several times a week). The panelists (n = 27 for first session, n=18 for second and third 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 ± 1°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 µg/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 µg/L, with about 15% of the wines ranging
376 between 30 and 40 µg/L. The mean and the median of the 98 wines were 18.7 µg/L and 13.6
377 µg/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 µg/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 µg/L with a mean of 0.91 µg/L. Furthermore, 1,8-cineole, another odorous marker of
388 green eucalyptus notes in red wines, presented concentrations that did not exceed 2.24 µg/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 ± 25 µg/L in the model wine solution. For each olfactory session, the
399 minimum detection threshold value by certain panel members was 9.6 µg/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 µg/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

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

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

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

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

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

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

3.4.1. Impact of downy mildew and other grape pathogens

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

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

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

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

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

3.4.2. Esca, grapevine trunk disease impact

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

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Notes

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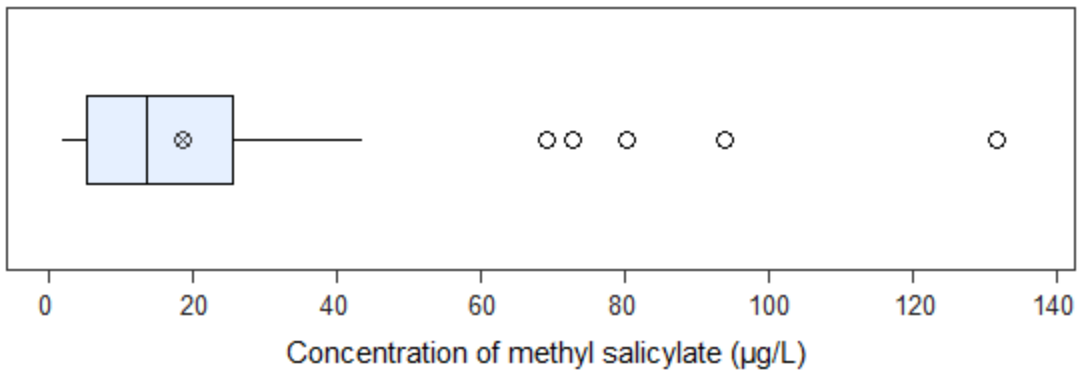
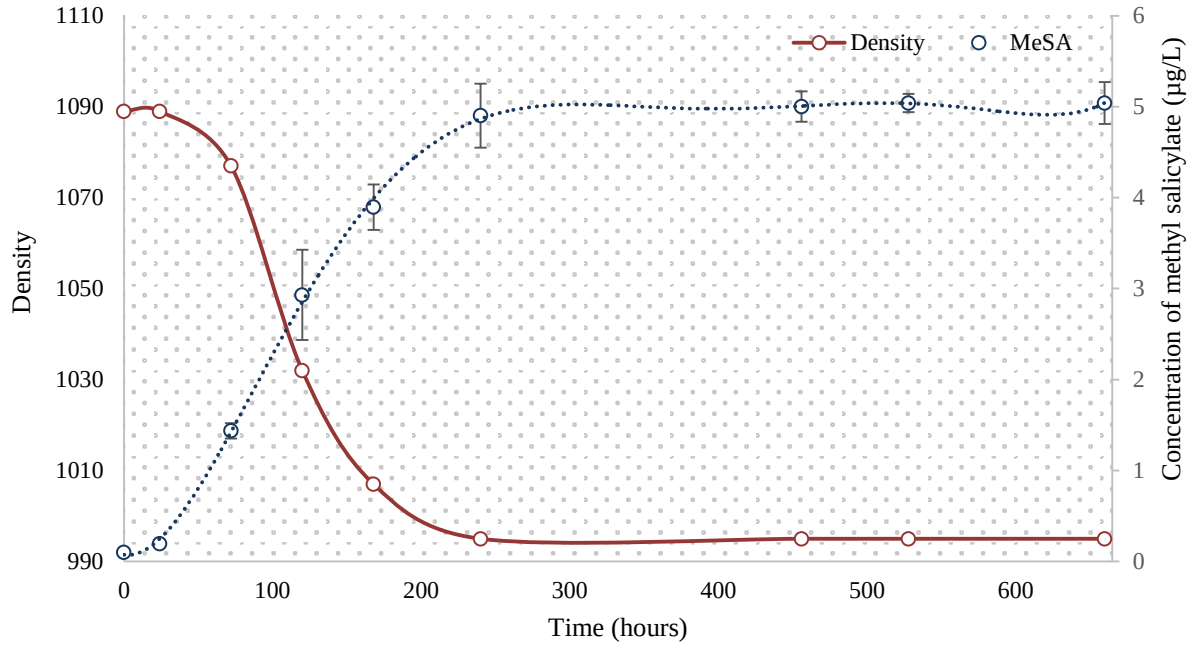

0
20
40
60
80
100
120
140
Concentration of methyl salicylate (µg/L)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MeSA
1110
1090
1070
1050
1030
1010
990
Density
0
100
200
300
400
500
600
Time (hours)
6
5
4
3
2
1
0
Concentration of methyl salicylate (µg/L)

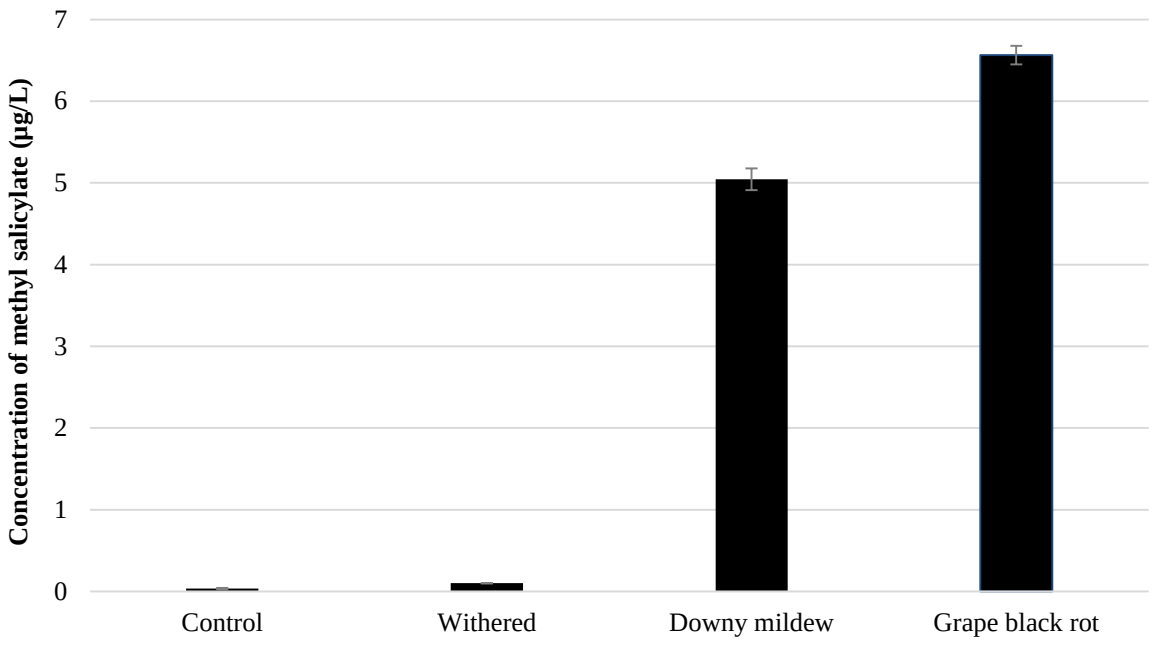
Concentration of methyl salicylate (μg/L)
7
6
5
4
3
2
1
0
Control
Withered
Downy mildew
Grape black rot

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

	Vintage	Volatile compounds [a]		
		Methyl salicylate (µg/L)	Ethyl salicylate (µg/L)	1,8-Cineole (µg/L)
Bordeaux (Merlot, on sand)	2006	**131.80 (19)**	0.79 (0.1)	0.07 (0.01)
Côtes du Rhône (Shiraz)	2014	**94.04 (0.5)**	2.82 (0.01)	0.2 (0.01)
Bordeaux (CS [b], Merlot blend)	2014	**80.36 (6.0)**	7.61 (0.5)	0.16 (0.02)
Bordeaux (Merlot, on gravels)	2006	72.94 (3.3)	0.74 (0.1)	0.05 (0.01)
Bordeaux (hard press wine)	2014	69.20 (1.0)	4.76 (0.1)	0.1 (0.01)
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 [b], Merlot blend)	2014	26.32 (1.5)	5.37 (0.3)	0.13 (0.01)
Pauillac (CS [b], Merlot blend)	2014	25.80 (4.0)	8.08 (2.0)	**1.04 (0.1)** [c]
Pauillac (CS, Merlot blend)	2009	25.67 (0.1)	9.81 (0.1)	**2.24 (0.03)**
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)

[a] For each compound, concentrations in bold corresponded to OAV (Odor Activity Value) >1.
[b] CS : Cabernet Sauvignon [c] Poitou et al. (2017)

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

	diseased berries (%) [b]					RC [c]
	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.

[a] Analysis done 6 years after bottling.

[b] Results concerning wines made with healthy grapes and grapes infected with increasing % of berries infected by brown rot (*P. viticola*)

[c] 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 (µg/L)
Cabernet Sauvignon (red wine, 2016)	Control	0.47 (0.25) [a] / 0.37 (0.1) [b]
	Esca level 1	1.25 (0.17) / 0.68 (0.05)
	Esca level 2	5.34 (0.93) / 1.48 (0.23)
Furmint (dry white wine, 2017)	Control	0.06 (0.02) [b]
	Incorporation level 1	0.16 (0.01)
	Incorporation level 2	0.42 (0.06)
	Incorporation level 3	0.62 (0.02)

[a] Concentration determined after 1 year ageing or [b] just after the end of alcoholic fermentation