



**HAL**  
open science

## Laccases 2 & 3 as biomarkers of *Botrytis cinerea* infection in sweet white wines

S. Ployon, A. Attina, J. Vialaret, Anne Sophie Walker, Christophe Hirtz,  
Cédric Saucier

► **To cite this version:**

S. Ployon, A. Attina, J. Vialaret, Anne Sophie Walker, Christophe Hirtz, et al.. Laccases 2 & 3 as biomarkers of *Botrytis cinerea* infection in sweet white wines. *Food Chemistry*, 2020, 315, pp.126233. 10.1016/j.foodchem.2020.126233 . hal-02505356

**HAL Id: hal-02505356**

**<https://hal.science/hal-02505356>**

Submitted on 21 Jul 2022

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License



11 **Abstract**

12 Botrytized sweet wines are made with berries infected by the fungus *Botrytis cinerea*. The aim  
13 of this study was to identify biomarkers of *B. cinerea* infection in sweet wines with a focus on laccases  
14 which are exocellular oxidase enzymes produced by this fungus during fruit contamination. Total  
15 proteins from six commercial sweet wines, including three naturally botrytized wines and three non-  
16 botrytized wines were analysed by LC-QTOF-MS. Five laccases, namely laccase-1-BcLCC1, laccase-  
17 2-BcLCC2, laccase-3-BcLCC7, laccase-8-BcLCC8 and laccase-12-BcLCC12, were identified in both  
18 types of wine. Then, a targeted proteomic approach by LC-MRM was used to semi-quantify laccase-2-  
19 BcLCC2 and laccase-3-BcLCC7, in the six samples. LC-MRM targeted analysis of the two enzymes  
20 allowed the discrimination of botrytized *versus* non-botrytized sweet white wines.

21 **KEYWORDS**

22 Botrytized white wines, *Botrytis cinerea*, noble rot, laccases

23

## 24 1. Introduction

25 Botrytized wines are sweet wines which are made with overripe berries from *Vitis vinifera*  
26 infected by the fungus *Botrytis cinerea* which are rich in aroma compounds (Tosi et al., 2012). They  
27 are produced in different countries in the world such as Hungary (Tokaji Aszu), Germany  
28 (Trockenbeerenauslese and Beerenauslese wines) and France (Sauternes) (Magyar, 2011; Teissedre &  
29 Donèche, 2013). Recently, the production of botrytized wines has increased in Australia, New  
30 Zealand, South Africa, and California (Magyar, 2011).

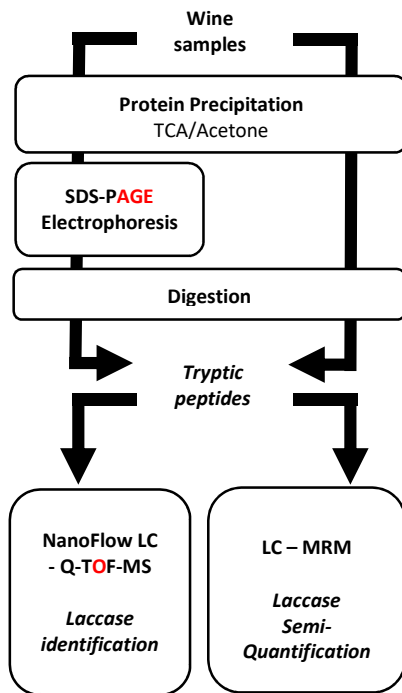
31 *Botrytis cinerea* (*Botryotinia fuckeliana*) is a filamentous and phytopathogenic fungus with a wide  
32 host range since it can infect more than 220 eudicot plants (Fournier, Gladieux, & Giraud, 2013). *B.*  
33 *cinerea* is notably the main pathogen infecting grapevines. Contamination of grape berries by *B.*  
34 *cinerea* was well compared to “Dr Jekyll and Mr Hyde” since its infection may take two very  
35 distinctive pathways, namely “noble rot” or “bunch rot”, closely depending on weather conditions  
36 (Fournier et al., 2013). The development of *B. cinerea* under mild climatic conditions (*i.e.* dry days  
37 followed by moisty nights) and a late grape harvest promotes noble rot development which induces  
38 favourable changes in berry composition. During the natural noble rot process, water evaporation  
39 through grape skin leads to a decrease of berry water content. Besides this process, many metabolic  
40 and physicochemical changes occur in the berries: an increase of sugar content due to berry  
41 dehydration, changes in sugar profile, changes in acid concentrations (Ribéreau-Gayon, Dubourdieu,  
42 & Donèche, 2006). Blanco-Ulate et al. (2015) reported an activation of the stilbene, flavonoid and  
43 anthocyanin biosynthesis in noble rot berries (Blanco-Ulate et al., 2015). The noble rot process  
44 impacts the berry aroma composition (Sarrazin, Dubourdieu, & Darriet, 2007) and especially its  
45 volatile thiol content (Thibon et al., 2010; Tominaga, Niclass, Frérot, & Dubourdieu, 2006), providing  
46 botrytized wines with a distinctive sensory profile with more grapefruit and passion fruit notes.

47 By contrast, a cool weather associated with heavy rainfalls favours *B.cinerea* development as  
48 bunch or grey rot in grape clusters. This leads to unwanted organoleptic properties in musts and wines  
49 such as the appearance of earthy mushroom aromas and other off-flavor compounds (Lopez Pinar,  
50 Rauhut, Ruehl, & Buettner, 2016; Steel, Blackman, & Schmidtke, 2013). In addition, the oxidation of

51 phenolic compounds by *B. cinerea* laccase enzymes leads to a decline of must and wine colour  
52 stability (Claus, 2017; Ribéreau-Gayon et al., 2006; Steel et al., 2013).

53 *B. cinerea* also impacts the grapes and wine proteomes as it secretes some enzymes and  
54 exhibits a high proteolytic activity leading to grape protein degradation (Cilindre, Castro, Clément,  
55 Jeandet, & Marchal, 2007; Kupfer, Vogt, Ziegler, Vogel, & Niessen, 2017; R. Marchal et al., 1998).  
56 This alteration of grape proteins affects wine properties and quality such as gushing (Kupfer et al.,  
57 2017) and foaming properties (Cilindre et al., 2007; Richard Marchal, Warchol, Cilindre, & Jeandet,  
58 2006). Infection is also characterised by production of *B. cinerea* proteins that can be found in berries  
59 and resulting wines, such as ATP synthase, hydroxysteroid dehydrogenases (Lorenzini et al., 2015),  
60 glycoside hydrolase, beta-1,3-gucanase (Perutka, Šufeisl, Strnad, & Šebela, 2019) and laccases  
61 (Kupfer et al., 2017; Perutka et al., 2019). Laccases (EC 1.10.3.2) are well-known and the most  
62 studied proteins of *B. cinerea* since they are responsible for major organoleptic problems and  
63 alteration of wine stability. They are highly glycosylated extracellular enzymes that belong to the  
64 family of polyphenol oxidase (PPO) (Claus, 2004). These multi-cooper oxidases are responsible for  
65 oxidative reactions in plants, using oxygen to oxidise a wide range of aromatic and non-aromatic  
66 substrates by a radical-catalysed reaction mechanism (Claus, 2004). In the case of grey mould  
67 contamination, the oxidation of phenolic compounds into highly reactive quinones that polymerize  
68 into brownish polymers responsible for wines colour alteration (Ribéreau-Gayon et al., 2006). The  
69 effect of laccase activity on organoleptic properties of musts and wines during noble rot infection is  
70 less clear. Even though laccase activity was detected in noble rot berries, its implication in the gold  
71 colour of botrytized wines was not elucidated and might be caused by glucose-oxidase activity more  
72 than laccase activity (Vivas et al., 2010). A recent study reported the presence of a single laccase in  
73 hazy white wines produced with partially botrytized grapes (Perutka et al., 2019). However, two  
74 laccases were identified by our research group from *in vitro* cultures of *B. cinerea* for three different  
75 strains (Quijada-Morin et al., 2017). To our knowledge, there was no study on laccases produced by *B.*  
76 *cinerea* in sweet white wines. The aim of our study is then to identify laccases present in botrytized  
77 white wines. First, a bottom-up qualitative proteomic approach was used to identify laccases present in

78 botrytized or non-botrytized sweet wines. Then, a targeted semi quantitative approach by LC-MRM  
79 was developed to semi-quantify the main laccases present in sweet white wines (Figure 1).



80

81 **FIGURE 1: Workflow**

82 **2. Material & Methods**

83 **2.1. Wine samples**

84 Three commercial non-botrytized sweet white wines (SWs) and 3 commercial botrytized wines (BWs)  
85 were used in this study. Samples are listed in table 1. After bottles opening, wines were divided into  
86 15 ml tube aliquots and kept at – 80°C.

87 **TABLE 1: List of wine samples used in the study, years, wine origin**

	<b>Sample name</b>	<b>Year</b>	<b>Appellation</b>
<b>Non-Botrytized Sweet Wins (SWs)</b>	SW1	2016	Jurançon
	SW2	2015	Alsace
	SW3	2014	Beaumes de Venise
<b>Botrytized Wins (BWs)</b>	BW1	2013	Sauternes
	BW2	2015	Sauternes
	BW3	2015	Sauternes

88

89 **2.2. Chemicals**

90 Acetone (analytic grade), trifluoroacetic acid (TFA) and dithiothreitol (DTT) were purchased from  
91 Sigma (Saint Quentin Fallavier, France). Trichloroacetic acid (TCA) was purchased from Acros  
92 Organics (Thermo Fisher). The reagents for sodium dodecyl sulfate polyacrylamide gel  
93 electrophoresis (SDS-PAGE) were purchased from Invitrogen. Urea was purchased from Merck  
94 KGaA (Darmstadt, Germany) and ammonium bicarbonate (ABC) was purchase from Fluka Analytical  
95 (Bucharest, Romania). Tris(hydroxymethyl)aminomethane (Tris) and iodoacetic acid (IAA) were  
96 purchased from VWR (Fontenay-sous-Bois, France). Water ULC-MS, formic acid ULC-MS (FA),  
97 acetonitrile ULC-MS (ACN), methanol ULC-MS (MeOH) were all from Biosolve (Dieuze, France).  
98 Trypsin Gold, Mass Spectrometry Grade, was purchased from Promega (Charbonnières-les-Bains,  
99 France).

100 **2.3. Trichloroacetic acid/acetone wine proteins extraction**

101 Total proteins from 10 ml of wines were precipitated overnight by adding 40 ml of 10% TCA/chilled  
102 acetone (–20°C). After precipitation, samples were centrifuged at 10 000 g for 20 min at 4°C. Proteins

103 pellets were washed twice with 1 ml of cold acetone and centrifuged at 10 000 g for 20 min at 4°C.  
104 Pellets were stored at – 80°C until use for Q-TOF and MRM analysis.

## 105 **2.4. Sample preparation**

### 106 **2.4.1. SDS-PAGE and in-gel protein digestion for Q-TOF protein identification**

107 Wine protein pellets were separated by SDS-PAGE electrophoresis using à 4-12 % Tris-Acetate  
108 Protein Gels (Invitrogen, Illkirch, France). Briefly, pellets were suspended in 50 µl of milliQ water, 26  
109 µl of the samples were mixed with 10 µl Novex NuPAGE LDS (4X) buffer (Invitrogen, Illkirch,  
110 France) and 4 µl of reducing agent NuPAGE10X (Invitrogen, Illkirch, France). Denaturation was  
111 performed 5 min at 90°C. Finally, samples were centrifuged to remove insoluble particles and loaded  
112 in SDS-PAGE gel with ready-to-use molecular-weight marker (SeeBlue® Plus 2 Prestained Standard  
113 1X (Invitrogen, Illkirch, France). Electrophoresis was carried out at 200 mA. Gel was stained with  
114 Blue Coomassie Instant Blue (Expedeon, San Diego, USA). For each wine sample, 8 bands were  
115 excised from the gel on a molecular weight range from 30 to 190 kDa. In gel digestion of bands was  
116 performed as following. Bands were washed successively twice with 150 µL of 50% ethanol in  
117 50mmol/L ABC (pH 8.4) and were dried using a Speedvac (Labconco, Kansas City, MO, USA). Then,  
118 the bands were successively reduced (10 mmol/L of DTT, 56°C;1 h), alkylated (55 mmol/L of IAA,  
119 37°C, 45 min), dehydrated (50% ethanol in 50 mmol/L ABC (pH 8.4)) and dried using a Speedvac.  
120 Bands were digested overnight at 37°C in 12.5 mg/mL of trypsin solution in 25 mmol/L ammonium  
121 carbonate. The digestion was stopped by adding 15 µL of pure FA (pH < 4). The tryptic peptides were  
122 desalted on BRAVO AssayMap (Agilent Technologies, Les Ulis, France) with C18 Tips conditioned  
123 with 50 µL of 70% ACN/0.1% FA and equilibrated with 50 µL of 0.1% FA. Loaded samples were  
124 washed twice with 50 µL of 0.1% FA and eluted with 50 µL of 70% ACN/0.1% FA. Samples were  
125 dried using a Speedvac (Labconco, Kansas City, MO, USA) and resuspended in 10 µL of phase A  
126 (0.1% formic acid, 2% ACN in water) for LC-MS/MS injections.

### 127 **2.4.2. Sample preparation for MRM analyses**

128 Protein pellets were obtained as described above. All the following steps were performed on Amicon  
129 Ultra 4, 30kDa centrifugal filter units (Merck Millipore, Molsheim, France). Protein pellets were



130 suspended and washed twice by 8 M urea in Tris-HCl (pH 8.5) and successively reduced in 10 mM of  
131 DTT in Tris-HCl (pH 8.5), alkylated in IAA in 0.1 M in Tris-HCl (pH 8.5), washed twice with 50 mM  
132 ABC buffer and then digested overnight (37°C) with trypsin at 1 µg/ml in 50 mM ABC buffer. Tryptic  
133 peptides were dried using a Speedvac (Thermo Fisher Scientific, Villebon sur Yvette, France) and  
134 resuspended in 30 µl ACN with 0.1% FA.

## 135 **2.5. Mass Analyses**

### 136 **2.5.1. Nano-Q-TOF-MS analyses for protein identification**

137 Seven microliters of the samples were injected in an HPLC NanoElute (Bruker, Billerica, USA)  
138 system coupled to a Q-TOF-MS instrument (Impact II, Bruker Daltonics, Champs sur Marne, France).  
139 Analyses were operated in nanoflow mode (400nL/min) NanoFlow LC. Samples were desalted and  
140 pre-concentrated on a PepMap u-precolumn (300 µm × 5 mm, C18 PepMap 100, 5 µm, 100 Å)  
141 (Thermo Fisher Scientific, Illkirch, France). The capillary pump was set at 20 µL/min and the mobile  
142 phase was 0.05% TFA, 2% ACN in water. Peptides were separated on a C18 analytical column (75  
143 µm × 500 mm; Acclaim Pepmap RSLC, C18, 2 µm, 100 Å). The peptides were eluted with a gradient  
144 consisting of 5–26% B for 45 min and 90% B for 4 min (A, 0.1% formic acid, 2% ACN in water; B,  
145 0.1% formic acid in ACN) at 400 nL/min. The temperature was set at 50°C. Peptides were identified  
146 by data-dependent acquisition (DDA). A lock-mass [m/z 1222, Hexakis (1H, 1H,  
147 4Hhexafluorobutyloxy) phosphazine] was used as the internal calibrator. Instant Expertise software  
148 (Bruker Daltonics) selected as many as possible of the most intense ions per cycle of 3 seconds and  
149 active exclusion was performed after one spectrum for 2 minutes only if the precursor ion exhibited an  
150 intensity three times greater than the previous scan. MS/MS spectra were searched against a  
151 SwissProt\_2017\_07\_28 database and against a home-made laccase short database adapted from Simon  
152 and Viaud (2018) (Simon & Viaud, 2018). Mascot v 2.4.1 algorithm (Matrix Science,  
153 <http://www.matrixscience.com/>) was used with the following settings: enzyme: trypsin; variable  
154 modifications: oxidation (M) and deamidated (N,Q); fixed modifications: carbamidomethyl (C);  
155 missed cleavages: 1; instrument type CID: ESI-QUAD-TOF; peptide tolerance: 10.0 ppm; MS/MS  
156 tolerance: 0.05 Da; peptide charge: 1+, 2+ and 3+; mass: monoisotopic; C13: 1; minimum peptide

157 length: 5; peptide decoy: ON; adjust FDR (%): 2.5; percolator: ON ; ions score cut-off: 12; ions score  
158 threshold for significant peptide IDs: 12.

## 159 **2.6. LC-MRM analyses for laccase targeted detection**

160 The two following peptides, YDSSSTVDPTSVGVTTPR (laccase 2) and YTTNTITAGK (laccase 3)  
161 were purchased from Eurogentec (Angers, France). Linearity of the two peptides was assessed.  
162 Synthetic standards were mixed and diluted in 0.1% FA (Phase A) at 1.4, 0.14 and 0.014 pmol/ $\mu$ L for  
163 YDSSSTVDPTSVGVTTPR and 0.14, 0.014 and 0.0014 pmol/ $\mu$ L for YTTNTITAGK. Each dilution  
164 was then injected at 1, 2, 4, 6 and 8  $\mu$ L in duplicates. Analyses were performed on LC (1290, Agilent  
165 Technologies, Les Ulis, France) – QqQ MS (6490, Agilent) system equipped with an AdvanceBio  
166 Peptide Plus column (2.1 x 150mm, 2.7 $\mu$ m) (Agilent Technologies, Les Ulis, France) maintained at  
167 56°C. The mobile phase was composed of 0.1% FA (Phase A) and ACN (Phase B). 20  $\mu$ l were  
168 injected and separated on 30 minutes' run with a non-linear gradient of 2.7 to 81 % of phase B at a  
169 flow rate of 400  $\mu$ l/min. The column was then washed for 1 min with 100% solvent B and re-  
170 equilibrated for 3 min with 15% solvent B. The ESI source was set as follows: capillary tension 3500  
171 V, nozzle voltage 300 V, nebulizer 30 psi, gas flow rate 15 l/min, gas temperature 150°C, sheath gas  
172 flow rate 11 l/min, sheath gas temperature 250°C. The MS was performed in dynamic MRM mode  
173 with a retention window of 1.5 min and a maximum cycle time fixed at 700 ms. Skyline software (v.  
174 1.4, <https://skyline.gs.washington.edu/>) was used to create a MRM transition list.  
175 YDSSSTVDPTSVGVTTPR peptide (MRM transition: 884.42 -> 913.51, fragmentor voltage: 380 V;  
176 Collision energy: 32 V; Cell accelerator voltage: 5 V; Retention time: 4 min) was monitored to detect  
177 the laccase-2-BcLCC2. YTTNTITAGK peptide (MRM transition: 535.28 -> 805.44, fragmentor  
178 voltage: 380 V; Collision energy: 12 V; Cell accelerator voltage: 5 V; Retention time: 4 min) was  
179 monitored to detect the laccase-3-BcLCC3. MS signal was integrated, manually checked, and exported  
180 to an Excel file with Skyline 4.1 (University of Washington). Analyses were performed on the same  
181 day in order to avoid inter-day variability on 3 biological replicates.

## 182 **2.7. Statistical analysis**

183 For MRM analyses, mean values of peak area are reported with their standard deviation. For each  
184 laccase, peptide peak area of the two groups BWs and SWs were compared by a Student t-test ( $\alpha =$   
185 0.05) using XLSTAT 2018.4 (Addinsoft, Paris, France).

186

187

188 **3. Results & Discussion**189 **3.1. Identification of laccases present in sweet wines by nano-LC-Q-TOF-MS**

190 Total proteins of a botrytized wine sample (BW1) and a non-botrytized wine sample (SW1) were  
 191 isolated by TCA/acetone precipitation and separated by SDS-PAGE electrophoresis. Tryptic digests  
 192 were analysed by nano-LC-MS and peptides were compared against two distinct databases with a  
 193 focus to identify laccases. In the molecular weight range studied (*i.e.* 30 to 190 kDa) fifteen proteins  
 194 were identified in the UniprotKB/SwissProt global protein database, mainly yeast protein from  
 195 *Saccharomyces cerevisiae* and enzymes from *B. cinerea* (Supp. Material S1).

196 A short homemade laccase database was established using laccase genes data collected by  
 197 Simon and Viaud (2018) to specifically identify laccases present in BW1 and SW1. Results are listed  
 198 in Table 2. Five different laccases were detected in the samples. Laccase-2-BcLCC2, laccase-3-  
 199 BcLCC7 and laccase-8-BcLCC8 were identified in both SW1 and BW1 samples. BcLCC12 was  
 200 detected only in SW1 while laccase-1-BcLCC1 was detected only in BW1.

201 **TABLE 2: MS identification table of laccases in sweet botrytized (BW1) or non-botrytized (SW1) wines using**  
 202 **Laccase short database (adapted from Simon & Viaud, 2018). Gene name, protein description, theoretical**  
 203 **molecular weight (Th. MW), Isoelectric point (pI), Mascot score (M. score), number of identified peptides,**  
 204 **and sequence coverage (SC) are reported.**

Sample	B0510-BROAD gene name	Usual gene name	Protein	Th. MW [kDa]	pI	M. Scores	#Peptides	SC [%]
SW1	BC1G_08553.1	<i>BcLcc2</i>	Laccase-2-BcLCC2	63.4	4.5	1101.5	18	26.7
	BC1G_10329.1	<i>BcLcc7</i>	Laccase-3-BcLCC7	71.1	5.7	64.6	1	1.5
	BC1G_14349.1	<i>BcLcc8</i>	Laccase-8-BcLCC8	76.4	5.4	23.6	1	1.3
	BC1G_15514.1	<i>BcLcc12</i>	Laccase-12- BcLCC12	67.1	5.2	15.5	1	2.6
BW1	BC1G_08553.1	<i>BcLcc2</i>	Laccase-2-BcLCC2	63.4	4.5	2074.0	23	26.7
	BC1G_10329.1	<i>BcLcc7</i>	Laccase-3-BcLCC7	71.1	5.7	407.6	9	16
	BC1G_14349.1	<i>BcLcc8</i>	Laccase-8-BcLCC8	76.4	5.4	51.6	1	1.3
	BC1G_00138.1	<i>BcLcc1</i>	Laccase-1-BcLCC1	60.8	5.9	30.8	1	1.8

205

206 Laccases were identified as isoenzymes in more than 115 fungus species (Baldrian, 2006).  
207 From a genetic point of view, 13 genes (*BcLcc1* to *BcLcc13*) encoding for laccases were identified in  
208 *B. cinerea* genome (Simon & Viaud, 2018).

209 Laccase-2-BcLCC2 is a 581 amino acids protein encoded by the *BcLcc2* gene. The molecular  
210 weight calculated for the enzyme is 63.4. A higher apparent molecular weight around 94 kDa was  
211 observed by SDS-PAGE electrophoresis likely due to glycosylation of these enzymes (Quijada-Morin  
212 et al., 2017). Laccase-2-BcLCC2 is the main laccase identified in *B. cinerea* cultures independently of  
213 the strain since studies reported its secretion by B0510 (González-Fernández et al., 2014; Quijada-  
214 Morin et al., 2017; Schouten, Wagemakers, Stefanato, van der Kaaij, & van Kan, 2002), RM344 and  
215 VA612 (Quijada-Morin et al., 2017) and T4 (González-Fernández et al., 2014) strains. Laccase-2-  
216 BcLCC2 was recently detected in hazy white wine (Perutka et al., 2019) and gushing sparkling white  
217 wine (Kupfer et al., 2017) produced with partially infected grapes. Laccase-2-BcLCC2 could be  
218 involved in detoxification of compounds contained in the plant, that are responsible for fungus  
219 development inhibition such as resveratrol (Schouten et al., 2002). Its ability to degrade the phenolic  
220 antibiotic 2,4-diacetylphloroglucinol was also demonstrated in vitro (Schouten, Maksimova, Cuesta-  
221 Arenas, van den Berg, & Raaijmakers, 2008).

222 Laccase-3-BcLCC7 is a 663 amino acids protein of 71.1 kDa encoded by the gene *BcLcc7*  
223 (Simon & Viaud, 2018). The expression of this laccase was reported for the first time by Quijada-  
224 Morin et al (2017) for three different *B. cinerea* strains, with slight differences in the amino acid  
225 sequence for the strains RM344 and VA612 compared to the reference strain B0510 (Quijada-Morin et  
226 al., 2017). In both cases, a lower number of peptides and a lower sequence coverage was observed  
227 compared to laccase-2-BcLCC2. To our knowledge, it is the first time that the laccase-3-BcLCC3 was  
228 identified in a wine matrix.

229 As far as we know, there are no proteomic study that report the presence of other three  
230 laccases in *B. cinerea* secretome. *BcLcc1* and *BcLcc8* genes expression was investigated by  
231 transcriptomic studies. *BcLcc1* transcriptome was not detected in *B. cinerea* cultures, even after

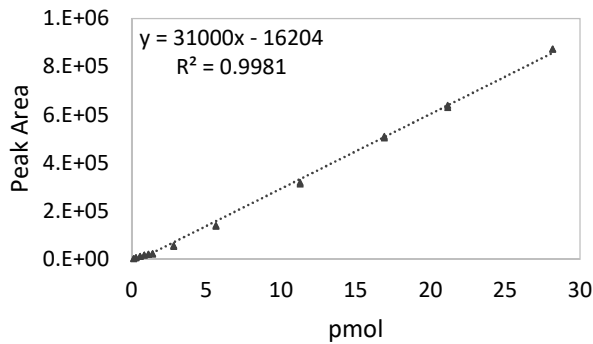
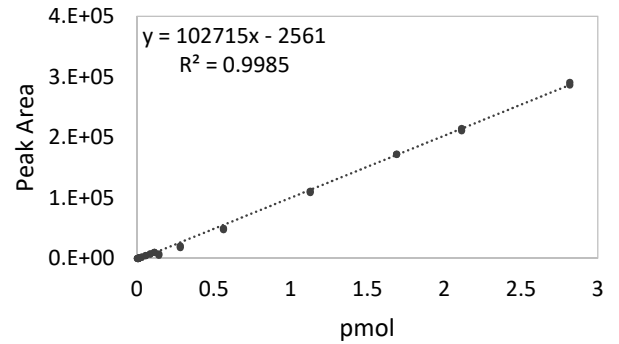
232 induction by phenolic compounds (Schouten et al., 2002). Regarding laccase-8-BcLCC8, an  
233 upregulation of *BcLcc8* gene during inoculation of tomatoes by *B. cinerea* was reported, but no protein  
234 expression was evidenced (Petrasch et al., 2019).

235 The abundance of MS fragments suggests that the amount of laccase-2-BcLCC2 is higher than  
236 other laccases in both wines. However, the Q-TOF results did not allow to discriminate both wines.  
237 Thus, a second approach was developed to provide a semi quantitative measurement of laccases in  
238 sweet white wines. Considering the high M. scores obtained for the identification of laccase-2-  
239 BcLCC2 and laccase-3-BcLCC7 (i.e. up to 400 for laccase-3-BcLCC7 and up to 2000 for laccase-2-  
240 BcLCC2 in BW, compared to values under 100 for laccase-1-BcLCC1, laccase-8-BcLCC8 and  
241 laccase-12-BcLCC12), we chose to focus on laccases-2-BcLCC2 and -3-BcLCC7 for the MRM semi-  
242 quantitative study.

### 243 **3.2. Multiple reaction monitoring (MRM) analysis for targeted detection of laccases-2- 244 BcLCC2 & laccase-3-BcLCC7**

245 The relative abundance of both laccase-2-BcLCC2 and laccase-3-BcLCC7 was investigated in  
246 three botrytized and three non-botrytized wines. Two peptides with a specific sequence for each  
247 enzyme were chosen: YDSSSTVDPTSVGVTTPR for laccase-2-BcLCC2 and YTTNTITAGK for  
248 laccase-3-BcLCC7. These peptides were then monitored by LC-MRM after tryptic digestion of the  
249 protein extracts.

250 Linearity of the MS signal of the two peptides was confirmed ( $R^2 > 0.99$ ) by using analytical  
251 standards in ACN. The peptide linearity ranges from 0.141 to 28.16 pmol for  
252 YDSSSTVDPTSVGVTTPR (Figure 2A) and from 0.0028 to 2.816 pmol for YTTNTITAGK (Figure  
253 2B). For YDSSSTVDPTSVGVTTPR (laccase 2), the LOD and LOQ calculated were 0.014 pmol/ $\mu$ L  
254 and 0.14 pmol/ $\mu$ L, respectively. For YTTNTITAGK (laccase 3), the LOD and LOQ calculated were  
255 0.0014 pmol/ $\mu$ L and 0.0028 pmol/ $\mu$ L, respectively.

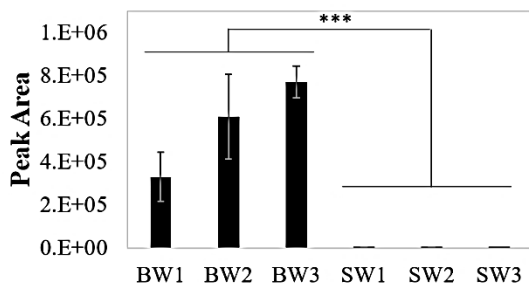
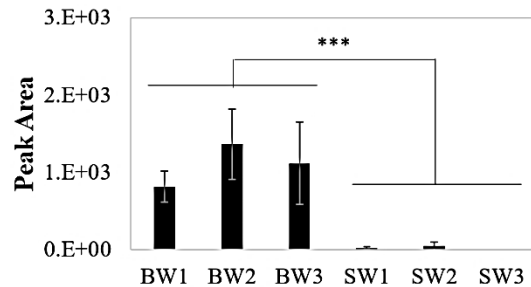
**A****B**

256

257 **FIGURE 2: Linearity of signals of the peptides (A) YDSSSTVDPTSVGVTPR (transition 884.42 -> 913.51)**  
 258 **and (B) YTTNTITAGK (transition 535.28 -> 805.44).**

259 The abundance of laccase-2-BcLCC2 and laccase-3-BcLCC7 peptides in three botrytized and  
 260 three non-botrytized wines are presented in figure 3 (A) and (B) respectively. Laccase-2-BcLCC2 was  
 261 detected in all samples BWs and SWs. The peak areas range from  $3.30 \cdot 10^5$  AU to  $7.70 \cdot 10^5$  AU in the  
 262 BWs and from  $0.16 \cdot 10^3$  AU to  $1.04 \cdot 10^3$  AU in the 3 SWs, indicating a significant greater laccase 2  
 263 concentration ( $p$ -value < 0.0001) in the 3 BWs compared to the 3 SWs.

264 Similarly, laccase-3-BcLCC7 was detected in the six wines samples. A significant difference  
 265 ( $p$ -value < 0.0001) was observed between BWs and SWs for their laccase 3 content. Peak areas were  
 266 significantly higher in BWs with value up to  $1.36 \cdot 10^3$  AU than in SWs, where peak area did not exceed  
 267  $0.05 \cdot 10^3$  AU. That represents more than a factor 27 for the intensities of the signals. Nevertheless, the  
 268 presence of both laccases in **non-botrytized** SWs could indicate a very minor contamination of grapes  
 269 used for their winemaking.

**A****B**

270

271 **FIGURE 3: Semi-quantification of laccase-2-BcLCC2 peptide (transition 884.42 -> 913.51) (A) and laccase-**  
272 **3-BcLCC7 peptide (transition 535.28 -> 805.44) (B) in botrytized sweet white wines (BWs) and non-botrytized**  
273 **sweet white wines (SWs). Significant difference between the two groups evaluated by Student t-test \*\*\* p-**  
274 **value < 0.0001 (Values are given in Supp. Material S2).**

275         The relative abundance of each peptide suggests that laccase-2-BcLCC2 was the major laccase  
276 present in all the wines. Generally, the composition of the growing environment of the fungus  
277 influences laccase production and some compounds may act as inducers of laccase secretion that can  
278 impact the laccase substrate specificity, molecular weight, glycosylation level and sugar pattern  
279 (Marbach, Harel, & Mayer, 1983). A previous study reported that laccase-2-BcLCC2 production was  
280 induced by gallic acid (Quijada-Morin et al., 2017). An increase of *BcLcc2* gene transcriptome was  
281 also evidenced in *B. cinerea* culture in presence of resveratrol and tannic acid (Schouten et al., 2002),  
282 that belong to the stilbenes and the tannins families, respectively, and in presence of cooper (Udugama  
283 Vithanage, Savocchia, Strappe, Schmidtke, & Steel, 2016). In accordance with our results, laccase-3-  
284 BcLCC7 seemed also to be minor in culture media in presence of gallic acid, suggesting the phenolic  
285 acid is not the main inducer of this laccase (Quijada-Morin et al., 2017).



286 **4. Conclusion**

287 Using an untargeted proteomic approach, five different laccases (laccase-1-BcLCC1, laccase-  
288 2-BcLCC2, laccase-3-BcLCC7, laccase-8-BcLCC8 and laccase-12-BcLCC12) were identified in  
289 sweet and botrytized wines. To our knowledge, this is the first time that the presence of laccase-1-  
290 BcLCC1, laccase-3-BcLCC7, laccase-8-BcLCC8 and laccase-12-BcLCC12 is reported in grape or  
291 wine.

292 Two laccases, namely laccase-2-BcLCC2 and laccase-3-BcLCC7 were semi-quantified in  
293 sweet and botrytized wines. Laccase-2-BcLCC2, which was also detected in bunch rot wines (Perutka  
294 et al., 2019) and culture media (Quijada-Morin et al., 2017; Schouten et al., 2002) seemed to be the  
295 main laccase produced by the fungus during grape infection. The second targeted laccase, laccase-3-  
296 BcLCC7, was detected in much lower amount.

297 This study revealed that laccase-2-BcLCC2 and laccase-3-BcLCC7 are still detectable in  
298 wines made with noble rot infected berries after several years of bottle ageing. Targeted detection by  
299 LC-MRM of the two laccases allowed the discrimination of botrytized from non-botrytized sweet  
300 white wines. These proteins are then very good quantitative biomarkers of *Botrytis* infection and are  
301 suitable for high throughput analysis of white wine quality in future studies.

302 **Conflict of interest statement**

303 The authors declare no conflict of interest.

304 **Acknowledgements**

305 We thank Mr Laurent Tiers for technical assistance. We thank the Biolaffort Company for funding.

306 **FIGURE CAPTIONS**

307 *FIGURE 1: Workflow*

308 *FIGURE 2: Linearity of signals of the peptides (A) YDSSSTVDPTSVGVTPR (transition 884.42 -> 913.51)*  
309 *and (B) YTTNTITAGK (transition 535.28 -> 805.44).*

310 *FIGURE 3: Semi-quantification of laccase-2-BcLCC2 peptide (transition 884.42 -> 913.51) (A) and laccase-*  
311 *3-BcLCC7 peptide (transition 535.28 -> 805.44) (B) in botrytized sweet white wines (BWs) and non-botrytized*  
312 *sweet white wines (SWs). Significant difference between the two groups evaluated by Student t-test \*\*\* p-*  
313 *value < 0.0001*

314 *TABLE 1: List of wine samples used in the study, years, wine origin*

315 *TABLE 2: MS identification table of laccases in sweet botrytized (BW1) or non-botrytized (SW1) wines using*  
316 *Laccase short database (adapted from Simon & Viaud, 2018). Gene name, protein description, theoretical*  
317 *molecular weight (Th. MW), Isoelectric point (pI), Mascot score (M. score), number of identified peptides,*  
318 *and sequence coverage (SC) are reported.*

319 *SUPPLEMENTARY MATERIAL S1: Total protein identification table*

320 *SUPPLEMENTARY MATERIAL S2: Transition results peptides YDSSSTVDPTSVGVTPR (transition*  
321 *884.42 -> 913.51) and YTTNTITAGK (transition 535.28 -> 805.44).*

322

323 **REFERENCES**

- 324 Baldrian, P. (2006). Fungal laccases – occurrence and properties. *FEMS Microbiology Reviews*, 30(2),  
325 215-242. <https://doi.org/10.1111/j.1574-4976.2005.00010.x>
- 326 Blanco-Ulate, B., Amrine, K. C., Collins, T. S., Rivero, R. M., Vicente, A. R., Morales-Cruz, A., ... Cantu,  
327 D. (2015). Developmental and metabolic plasticity of white-skinned grape berries in response  
328 to *Botrytis cinerea* during noble rot. *Plant Physiology*, pp.00852.2015.  
329 <https://doi.org/10.1104/pp.15.00852>
- 330 Cilindre, C., Castro, A. J., Clément, C., Jeandet, P., & Marchal, R. (2007). Influence of *Botrytis cinerea*  
331 infection on Champagne wine proteins (characterized by two-dimensional  
332 electrophoresis/immunodetection) and wine foaming properties. *Food Chemistry*, 103(1),  
333 139-149. <https://doi.org/10.1016/j.foodchem.2006.07.043>
- 334 Claus, H. (2004). Laccases : Structure, reactions, distribution. *Micron*, 35(1-2), 93-96.  
335 <https://doi.org/10.1016/j.micron.2003.10.029>
- 336 Claus, H. (2017). Laccases of *Botrytis cinerea*. In H. König, G. Uden, & J. Fröhlich (Éd.), *Biology of*  
337 *Microorganisms on Grapes, in Must and in Wine* (p. 339-356). [https://doi.org/10.1007/978-3-](https://doi.org/10.1007/978-3-319-60021-5_14)  
338 [319-60021-5\\_14](https://doi.org/10.1007/978-3-319-60021-5_14)
- 339 Fournier, E., Gladioux, P., & Giraud, T. (2013). The ‘Dr Jekyll and Mr Hyde fungus’ : Noble rot versus  
340 gray mold symptoms of *Botrytis cinerea* on grapes. *Evolutionary Applications*, 6(6), 960-969.  
341 <https://doi.org/10.1111/eva.12079>
- 342 González-Fernández, R., Aloria, K., Valero-Galván, J., Redondo, I., Arizmendi, J. M., & Jorrín-Novó, J.  
343 V. (2014). Proteomic analysis of mycelium and secretome of different *Botrytis cinerea* wild-  
344 type strains. *Journal of Proteomics*, 97, 195-221. <https://doi.org/10.1016/j.jprot.2013.06.022>
- 345 Kupfer, V. M., Vogt, E. I., Ziegler, T., Vogel, R. F., & Niessen, L. (2017). Comparative protein profile  
346 analysis of wines made from *Botrytis cinerea* infected and healthy grapes reveals a novel  
347 biomarker for gushing in sparkling wine. *Food Research International*, 99, 501-509.  
348 <https://doi.org/10.1016/j.foodres.2017.06.004>

349 Lopez Pinar, A., Rauhut, D., Ruehl, E., & Buettner, A. (2016). Effects of *Botrytis cinerea* and *Erysiphe*  
350 *necator* fungi on the aroma character of grape must : A comparative approach. *Food*  
351 *Chemistry*, 207, 251-260. <https://doi.org/10.1016/j.foodchem.2016.03.110>

352 Lorenzini, M., Millionini, R., Franchin, C., Zapparoli, G., Arrigoni, G., & Simonato, B. (2015).  
353 Identification of potential protein markers of noble rot infected grapes. *Food Chemistry*, 179,  
354 170-174. <https://doi.org/10.1016/j.foodchem.2015.01.112>

355 Magyar, I. (2011). Botrytized Wines. In *Advances in Food and Nutrition Research* (Vol. 63, p. 147-206).  
356 <https://doi.org/10.1016/B978-0-12-384927-4.00006-3>

357 Marbach, I., Harel, E., & Mayer, A. M. (1983). Inducer and culture medium dependent properties of  
358 extracellular laccase from *Botrytis cinerea*. *Phytochemistry*, 22(7), 1535-1538.  
359 [https://doi.org/10.1016/0031-9422\(83\)80083-1](https://doi.org/10.1016/0031-9422(83)80083-1)

360 Marchal, R., Berthier, L., Legendre, L., Marchal-Delahaut, L., Jeandet, P., & Maujean, A. (1998). Effects  
361 of *Botrytis cinerea* Infection on the Must Protein Electrophoretic Characteristics. *Journal of*  
362 *Agricultural and Food Chemistry*, 46(12), 4945-4949. <https://doi.org/10.1021/jf980453b>

363 Marchal, Richard, Warchol, M., Cilindre, C., & Jeandet, P. (2006). Evidence for Protein Degradation by  
364 *Botrytis cinerea* and Relationships with Alteration of Synthetic Wine Foaming Properties.  
365 *Journal of Agricultural and Food Chemistry*, 54(14), 5157-5165.  
366 <https://doi.org/10.1021/jf060509z>

367 Perutka, Z., Šufeisl, M., Strnad, M., & Šebela, M. (2019). High-proline proteins in experimental hazy  
368 white wine produced from partially botrytized grapes : High-Proline Proteins in Hazy White  
369 Wine. *Biotechnology and Applied Biochemistry*. <https://doi.org/10.1002/bab.1736>

370 Petrasch, S., Silva, C. J., Mesquida-Pesci, S. D., Gallegos, K., van den Abeele, C., Papin, V., ... Blanco-  
371 Ulate, B. (2019). Infection Strategies Deployed by *Botrytis cinerea*, *Fusarium acuminatum*,  
372 and *Rhizopus stolonifer* as a Function of Tomato Fruit Ripening Stage. *Frontiers in Plant*  
373 *Science*, 10, 223. <https://doi.org/10.3389/fpls.2019.00223>

374 Quijada-Morin, N., Garcia, F., Lambert, K., Walker, A.-S., Tiers, L., Viaud, M., ... Saucier, C. (2017).  
375 Strain effect on extracellular laccase activities from *Botrytis cinerea* : Strain effect on laccases  
376 from *Botrytis cinerea*. *Australian Journal of Grape and Wine Research*.  
377 <https://doi.org/10.1111/ajgw.12322>

378 Ribéreau-Gayon, P., Dubourdieu, D., & Donèche, B. (2006). *Handbook of enology* (2nd ed).  
379 Chichester, West Sussex, England ; Hoboken, NJ: John Wiley.

380 Sarrazin, E., Dubourdieu, D., & Darriet, P. (2007). Characterization of key-aroma compounds of  
381 botrytized wines, influence of grape botrytization. *Food Chemistry*, *103*(2), 536-545.  
382 <https://doi.org/10.1016/j.foodchem.2006.08.026>

383 Schouten, A., Maksimova, O., Cuesta-Arenas, Y., van den Berg, G., & Raaijmakers, J. M. (2008).  
384 Involvement of the ABC transporter BcAtrB and the laccase BcLCC2 in defence of *Botrytis*  
385 *cinerea* against the broad-spectrum antibiotic 2,4-diacetylphloroglucinol. *Environmental*  
386 *Microbiology*, *10*(5), 1145-1157. <https://doi.org/10.1111/j.1462-2920.2007.01531.x>

387 Schouten, A., Wagemakers, L., Stefanato, F. L., van der Kaaij, R. M., & van Kan, J. A. L. (2002).  
388 Resveratrol acts as a natural antifungicide and induces self-intoxication by a specific laccase.  
389 *Molecular Microbiology*, *43*(4), 883-894.

390 Simon, A., & Viaud, M. (2018). *Cross-reference table for Botrytis cinerea B0510 and T4 gene ids* [Data  
391 set]. <https://doi.org/10.15454/ihyjcx>

392 Steel, C. C., Blackman, J. W., & Schmidtke, L. M. (2013). Grapevine Bunch Rots : Impacts on Wine  
393 Composition, Quality, and Potential Procedures for the Removal of Wine Faults. *Journal of*  
394 *Agricultural and Food Chemistry*, *61*(22), 5189-5206. <https://doi.org/10.1021/jf400641r>

395 Teissedre, P.-L., & Donèche, B. (2013). Botrytized Wines : Sauternes, German Wines. In F. Mencarelli  
396 & P. Tonutti (Éd.), *Sweet, Reinforced and Fortified Wines* (p. 285-299).  
397 <https://doi.org/10.1002/9781118569184.ch19>

398 Thibon, C., Shinkaruk, S., Jourdes, M., Bennetau, B., Dubourdieu, D., & Tominaga, T. (2010). Aromatic  
399 potential of botrytized white wine grapes : Identification and quantification of new cysteine-

400 S-conjugate flavor precursors. *Analytica Chimica Acta*, 660(1-2), 190-196.  
401 <https://doi.org/10.1016/j.aca.2009.10.018>

402 Tominaga, T., Niclass, Y., Frérot, E., & Dubourdieu, D. (2006). Stereoisomeric Distribution of 3-  
403 Mercaptohexan-1-ol and 3-Mercaptohexyl Acetate in Dry and Sweet White Wines Made  
404 from *Vitis vinifera* (Var. Sauvignon Blanc and Semillon). *Journal of Agricultural and Food*  
405 *Chemistry*, 54(19), 7251-7255. <https://doi.org/10.1021/jf061566v>

406 Tosi, E., Fedrizzi, B., Azzolini, M., Finato, F., Simonato, B., & Zapparoli, G. (2012). Effects of noble rot  
407 on must composition and aroma profile of Amarone wine produced by the traditional grape  
408 withering protocol. *Food Chemistry*, 130(2), 370-375.  
409 <https://doi.org/10.1016/j.foodchem.2011.07.053>

410 Udegama Vithanage, A., Savocchia, S., Strappe, P., Schmidtke, L. M., & Steel, C. C. (2016). *Copper*  
411 *induction of laccases in the plant pathogenic fungus Botrytis cinerea*. 122. Brisbane  
412 Convention Center.

413 Vivas, N., Vivas de Gaulejac, N., Vitry, C., Bourden-Nonier, M. F., Chauvert, S., Doneche, B., ...  
414 Mouche, C. (2010). Occurrence and specificity of glucose oxidase (E.C: 1.1.3.4) in botrytized  
415 sweet white wine. Comparison with laccase (E.C: 1.10.3.2), considered as the main  
416 responsible factor for oxidation in this type of wine. *Vitis*, p. 113-120.  
417  
418