Performance of the extremophilic enzyme *BglA* in the hydrolysis of two aroma glucosides in a range of model and real wines and juices.

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ABSTRACT

β-Glycosidases enhance wine aroma by releasing volatile aglycones from non-volatile glycosides. Commercial preparations contain primarily pectinases and only display β-glycosidase as a secondary activity which limits their potential. Here, the extremophilic β-glucosidase A from *Halothermothix orenii*, (*BglA*) has been compared with Rapidase® for the production of aromatic wines and in the remediation of smoke-tainted wines. Model systems as well as real juices and wines have been enriched with geranyl glucoside, typical of white varieties, and guaiacyl glucoside, commonly found in red wines exposed to oak and wines made from grapes exposed to smoke. The hydrolytic capacity

of *BglA* was evaluated by measuring the released volatiles in the gas phase with Solid Phase Microextraction and GC-MS. *BglA*, despite an apparent instability at low pHs, is twice as effective in the release of volatiles in sweeter wines and in grape juices offering an excellent alternative for the early stages of the winemaking process and in the juice industry.

Keywords: Enzyme, Glucosides, Wine, Monoterpenes, Aroma, GC-MS, SPME

1 Introduction

Aroma is considered a key aspect of wine quality. Despite the identification of over 800 aroma compounds (Rapp, 1990) only a small number of them contribute substantially to the aroma of wine (Francis & Newton, 2005; Loscos, Hernández-Orte, Cacho, & Ferreira, 2009). Among the volatiles that are important to the aroma of wine there are fruity and floral monoterpenes (geraniol, linalool and a-terpineol) and volatile phenols (guaiacol and cresols), which, depending on their concentration and wine style, could affect differently the overall flavour and aroma.

Monoterpenes, formed in grapes during ripening, are crucial components of the varietal wine bouquet of Muscat and floral varieties (Sánchez Palomo, Pérez-Coello, Díaz-Maroto, González Viñas, & Cabezudo, 2006) but a major fraction is entrapped as flavourless, odourless, non-volatile glycosides, constituting an important reservoir of aroma (Skouroumounis, Massy-Westropp, Sefton, & Williams, 1995). Monoterpenes can be liberated from their glycosides by acid or enzymatic hydrolysis; but as acid hydrolysis is a slower process (Mojsov, Andronikov, Janevski, Jordeva, & Zezova, 2015; Wilkowska & Pogorzelski, 2017) and can cause rearrangements of the released aglycones, enzymes represent a useful alternative and can be added to maximize the aromatic potential of wines (González-Pombo, Fariña, Carrau, Batista-Viera, & Brena, 2014; Günata, Dugelay, Sapis, Baumes, & Bayonove 1993).

Phenolic glycosides are also formed when berries are exposed to smoke from bush fires and prescribed forest burns as the grapevines can uptake smoke constituents like guaiacols, cresols and

50 syringols, and accumulate them in the form of glycoconjugates. However in this case, their hydrolysis
51 leads to the release of volatile phenols (VP) giving the wine a “smoky” or “ashy” aroma/flavour
52 (Hayasaka, Dungey, Baldock, Kennison, & Wilkinson, 2010; Mayr et al., 2014; Singh et al., 2011).
53 In addition, breakdown of glycosides of volatile phenols in the mouth, mediated by enzymes of the
54 oral microflora can also contribute to smoky and ashy aftertaste (Parker et al., 2012). In this case, if
55 enzymatic hydrolysis can be performed effectively during the wine processing, phenolic glycosides
56 can be reduced, and the release of VPs can then be minimised using different techniques (van der
57 Hulst et al., 2019), improving the overall flavour.

58 The aglycone moiety in terpenyl and phenol glycosides can be linked to a β-D-glucopyranose unit or
59 to a disaccharide (Hjelmeland & Ebeler, 2015). While β-glucosidases [E.C.3.2.1.21] are capable of
60 cleaving the glycosidic bond between the carbohydrate moiety and the aglycone (Singh, Verma, &
61 Kumar, 2016), the release of the aglycone from disaccharide glycosides would normally require the
62 action of other glycosyl hydrolases. Endogenous glycosidases from the grape and the winery
63 environment have been extensively studied for this purpose; however, they do not tolerate well the
64 harsh physical and chemical conditions that usually characterize wine processing such as low pH,
65 high glucose and fructose, and sulphite content. Grape and yeast glycosidases present low activity
66 under fermentation conditions (Sánchez Palomo, Díaz-Maroto Hidalgo, González-Viñas, & Pérez-
67 Coello, 2005), therefore commercial preparations are mainly obtained from fungi and have primarily
68 pectinase activity, with secondary glycosidase activity. Fungal glycosidases have a weak catalytic
69 specificity which could lead to the hydrolysis of pigment glycosides, and consequent spoiling of
70 colours and flavours (Hu et al., 2016). In addition, glucose inhibition is a common problem among
71 fungal β-glucosidases (Chan et al., 2016; Maicas & Mateo, 2005; Sabel, Martens, Petri, König, &
72 Claus, 2014). Hence, the search for new enzymatic alternatives, more adapted to the wine conditions,
73 is highly relevant.

74 Extremophiles, organisms very well adapted to extreme environmental conditions unbearably hostile
75 or even lethal for other forms of life (Rampelotto, 2013), constitute a novel and alternative source of

76 enzymes for industrial application. Extremozymes are generally more capable to withstand industrial
77 processes in comparison with their mesophilic counterparts (Elleuche, Schröder, Sahm, &
78 Antranikian, 2014). Among extremophiles, enzymes from halophilic microorganisms tolerate very
79 high salinity, which normally leads to denaturation, aggregation, and precipitation of most other
80 proteins. Genomic and structural analyses have established that halophilic enzymes have a higher
81 pro-ratio of acidic amino acids versus hydrophobic ones and altered hydrophobicity compared to
82 mesophilic enzymes, which enhance solubility and promote function in low water activity conditions
83 (DasSarma & DasSarma, 2015). Adaptation to solvents follows the same principle as adaptation to
84 salt, and thus, halophilic enzymes may be a valid option for biocatalytic processes performed in
85 water/solvent environments like wines (Alsafadi & Paradisi, 2013).

86 Based on this hypothesis, the extremophilic organism *Halothemothrix orenii* was selected as a source
87 of a β-glucosidase for possible application in the wine industry. *Halothemothrix orenii* is a true
88 halophilic and thermophilic bacterium whose unique enzymes are described to have broad pH
89 stability and ability to deal with high temperatures and a wide range of salt concentrations
90 (Bhattacharya & Pletschke, 2014). In this work we evaluated the hydrolytic performance of the β-
91 glucosidase *BglA* described by Kori et al. (Kori, Hofmann, & Patel, 2011) with two glucosides
92 relevant to floral wine aroma and smoke-taint affected wines and compared it with a commercial
93 preparation (Rapidase® Revelation Aroma).

94 2 Materials and methods

95 *2.1 Chemicals*

96 Water was obtained from a Milli-Q purification system (Millipore, North Ryde, NSW, Australia).
97 Luria Bertani Broth, Miller and LB Agar, Miller were purchased from Fisher BioReagents™,
98 Imidazole 99 % was purchased from Alfa Aesar (Fisher Scientific, Bishop Meadow Road,
99 Loughborough, UK). Citric acid monohydrate, potassium L-tartrate monobasic, D-(+)-glucose 99.5

100 %, D-(-)-fructose European Pharmacopoeia 98% and DL-malic acid ≥ 98 % (capillary GC) were
101 purchased from Sigma-Aldrich (Castle Hill, New South Wales, Australia). Hepes ≥ 99.5 % (titration),
102 sodium chloride, acetone Suprasolv® ECD, ethanol for liquid chromatography LiChrosolv®, tartaric
103 acid and *p*-nitrophenyl β-D-glucopyranoside (*p*NPG) were obtained from Merck Pty Ltd (Kilsyth,
104 Victoria, Australia). Rapidase® Revelation Aroma enzymatic preparation was purchased from
105 Vintessential Laboratories (Dromana, Victoria, Australia). Geranyl glucoside, guaiacyl glucoside, d_7-
106 geraniol and d_3-guaiacol were synthesised in-house (Hayasaka et al., 2010; Parker et al., 2012;
107 Pedersen, Capone, Skouroumounis, Pollnitz, & Sefton, 2003; Pollnitz, Pardon, Sykes, & Sefton,
108 2004).

109 *2.2 Microbial strains*

110 The halothermophilic microorganism *Halothermothrix orenii* H 168 was the source of the native β-
111 glucosidase family 1 *BglA*. The constructed vector (BglA-pET45b) was kindly provided to us by Prof.
112 J. Siegel at UC Davis. *E. coli* BL21 (DE3) was the laboratory strain chosen for the heterologous
113 expression.

114 *2.3 Enzyme expression, purification and lyophilisation*

115 Cells of *E. coli* BL21 (DE3) harbouring the recombinant plasmid were grown at 37 ºC in Luria-
116 Bertani medium supplemented with ampicillin (0.1 mg/mL). When the OD_{600} was between 0.6-0.8,
117 isopropyl β-D-1-thiogalactopyranoside was added as inductor for the overexpression of the enzyme
118 and the culture left at 30 ºC overnight. Cells were harvested at 4500 G, 4 °C, 20 min and the pellet
119 stored at -20 °C until purification.

120 The cell pellet was resuspended in buffer (HEPES (50 mM), sodium chloride (150 mM), imidazole
121 (10 mM), pH 7.5) and cells were broken by sonication (6 min cycle, 5s on, 5s off, 50 % amplification).
122 The lysate was collected by centrifugation at 14500 G, 1 h, 4º C, and the pellet was discarded.

123 The supernatant was then filtered through Millex® PVDF 0.45 µm filter before loading it onto a
124 HisTrap IMAC column previously loaded with $NiSO_4$ 0.1 M and washed with loading buffer (HEPES

125 (50 mM), sodium chloride (150 mM), imidazole (10 mM), pH 7.5). The column was washed with
126 loading buffer until a plateau in the UV_{280} absorbance was reached. Low affinity binding proteins
127 were eluted using a step gradient 10 % elution buffer and the protein of interest was eluted using 100
128 % elution buffer (HEPES (50 mM), sodium chloride (150 mM), imidazole (300 mM), pH 7.5). The
129 enzyme was dialysed overnight, flash frozen in liquid nitrogen and freeze dried overnight. (Labconco
130 8 Port Manifold on Consolo Freeze Dryer).

131 *2.4 Protein quantification and SDS-PAGE*

132 Bradford Protein Assay was used for protein quantification using bovine serum albumin as standard.
133 Sodium dodecyl sulphate electrophoresis was performed to assess protein purity. Image Studio
134 Software (version 4.0) was used to quantify the size of the bands corresponding to the proteins of
135 interest.

136 *2.5 Activity test*

137 β-glucosidase activity was determined spectrophotometrically by adding 10 µL of the suitable
138 enzyme dilution and 290 µL of 10 mM *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) in buffer HEPES
139 50 mM, pH 7.4 at 25 ºC. The specific activity (U/mg) was expressed as µmol of product formed per
140 minute per milligram of protein.

141 *2.6 Model wines and juices*

142 Two different model wines were selected in representation of a completely sugar dry wine and a table
143 wine with sugar concentrations typical for Australian commercial wines (Godden, Wilkes, &
144 Johnson, 2015). Model wine 1 (MW1) consisted of saturated potassium hydrogen tartrate with 10 %
145 (v/v) ethanol, pH 3.5. Model wine 2 (MW2) consisted of saturated potassium hydrogen tartrate with
146 10% (v/v) ethanol, 6 g/L glucose, 6 g/L fructose, pH 3.5.

147 Model juice (MJ) was prepared using water, 100 g/L glucose, 100 g/L fructose, 0. 2 g/L citric acid, 3
148 g/L malic acid, 2.5 g/L tartaric acid, pH 3.7. pH was adjusted with tartaric acid 1M in all cases.

149 *2.7 Real wines and juices*

150 Two commercially available wines, one white (WW) and one red (RW), and a Chardonnay grape
151 juice (WJ) produced in-house were used. A 2017 Chardonnay from Riverina, Australia with an
152 alcohol content of 12.2% v/v, 4.9 g/L glucose and fructose, titratable acid 6.4 g/L and pH 3.35, a 2016
153 Shiraz from South Eastern Australia with an alcohol content of 13.9% v/v, 5.8 g/L glucose and
154 fructose, titratable acid 6.2 g/L and pH 3.66 and a Chardonnay juice with total soluble solids 22.6
155 ºBrix (~20 % total sugar content), 52 mg/L SO_2 and pH 3.5. Chardonnay and Shiraz grape varieties
156 were chosen due to their low monoterpene content.

157 *2.8 Enzymatic treatment*

158 In separate 20 mL SPME vials, 3 mL of MW1, MW2, MJ, WW, RW and WJ were spiked with 5 µg
159 of geranyl glucoside and 5 µg of guaiacyl glucoside. The amount added to each sample of Rapidase®
160 or *BglA* was 0.01 mg/mL. The samples were left shaking at 22 ºC over different incubation periods
161 to allow enzymatic hydrolysis. The reaction was stopped by adding 2 mL of saturated $CaCl_2$. Internal
162 standards, d_7-geraniol and d_3-guaiacol, were added (2 µg) and the liberated aglycones were analysed
163 using SPME-GCMS. All experiments were carried out in triplicate.

164 Geraniol and guaiacol calibration curves with a linear range between 0.02-5 µg were performed for
165 each matrix.

166 *2.9 GC-MS analysis of volatiles*

167 A Gerstel autosampler (MPS) (Lasersan Australasia Pty Ltd, Robina, Queensland, Australia) was
168 fitted with a 2 cm DVB/CAR/PDMS fibre assembly (Supelco, Bellefonte, PA) to sample the
169 headspace above the stirred sample for 20 min at 35 ºC, immediately prior to instrumental analysis.
170 Analyses were carried out with an Agilent 6890A gas chromatograph and an Agilent 5973 mass
171 selective detector (Agilent Technologies, Forest Hill, Australia) fitted with a Gerstel autosampler
172 (MPS). The sample was injected in splitless mode. The splitter, at 58:1, was opened after 60 s. The
173 injection liner was a Supelco injection sleeve made of 0.75 mm i.d. deactivated borosilicate glass.

174 The gas chromatograph was fitted with a 30 m x 0.25 mm Agilent J&W DB-35ms Ultra Inert column,
175 0.25 µm film thickness. The carrier gas was helium, linear velocity was 36 cm/s, and flow rate was 1
176 mL/min. The oven temperature, was held at 40 ºC for 1 min, increased to 240 ºC at a 5 ºC/min rate,
177 and held at this temperature for 2 min. The injector temperature was 220 ºC, and the transfer line was
178 held at 240 ºC. Positive electron ionisation mass spectra at eV were recorded in SIM mode with *m/z*
179 69, 81, 93, 99, 109, 121, 123, 124, 127, 128, 136, 154, and 161 with dwell 25 ms (See section 1 of
180 the supplementary information for geraniol and guaiacol quantifiers and qualifiers for identification
181 with MS).

182 Mass Hunter software (version B.09.00 Agilent) was used for the quantitative analysis.

183 The hydrolysis percentages were calculated using the following equations:

184 $$\%\ geraniol\ release = \left(\left(\frac{amount\ of\ free\ geraniol\ detected}{amount\ of\ geranyl\ glucoside\ added}\right)\frac{316}{154}\right) \times 100$$

185 $$\%\ guaiacol\ release = \left(\left(\frac{amount\ of\ free\ guaiacol\ detected}{amount\ of\ guaiacyl\ glucoside\ added}\right)\frac{286}{124}\right) \times 100$$

186 *2.10 Data analysis*

187 For the experiments in model wines (MW1, MW2) and model juice (MJ) two-way analyses of
188 variance (ANOVA) (GraphPad Prism 8, San Diego, California, USA) were carried out to assess the
189 effects of enzyme and incubation period on the hydrolysis of glycosides. For the experiments in real
190 wines (WW, RW) and real juice (WJ) a paired t-test was run to assess the effect of the enzyme.
191 Significant difference values were calculated in all cases (****$\rho \leq 0.0001$; ***$\rho \leq 0.001$; **$\rho \leq 0.01$,
192 *$\rho < 0.05$).

3 Results and discussion

3.1 Protein expression, purification, and lyophilisation.

BglA was expressed with an average yield of 53 mg protein/L of culture. Estimation by quantification analysis (using Li-cor Odyssey Fc scanner and software Image Studio version 4.0) suggested that 13 % of the crude extract corresponded to *BglA* (Fig 1). The activity of the crude extract was found to be 2.1 U/mg of total proteins. The enzyme was then purified by metal affinity chromatography, to better asses its hydrolytic capacity, and SDS-PAGE was done to assess its purity. For Rapidase®, estimation by quantification suggests that 10 % of the commercial preparation would correspond to β-glucosidases (Fig 1).

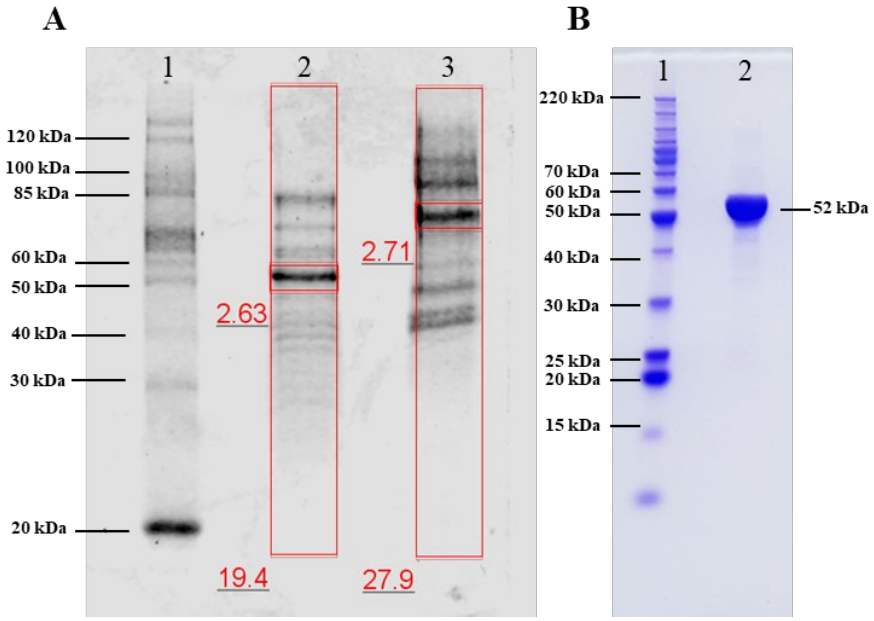


Figure 1. A. Quantification of the bands corresponding to *BglA* in the crude extract and to the β-glucosidase in the Rapidase® preparation are indicated in red, the signal is expressed in relative fluorescence units (RFU) using Li-cor Odyssey Fc scanner and software Image Studio version 4.0), (1) ThermoFisher Scientific PageRuler™ Unstained Protein Ladder, (2) *BglA* in the crude extract (5 µg) (3) β-glucosidases in Rapidase® (5 µg). B. SDS-PAGE after *BglA* purification (1) Invitrogen™ BenchMar™ Protein Ladder (2) Pure *BglA* (5 µg).

Following dialysis, *BglA* was lyophilized and stored at 4 ºC until needed. An activity assay under standard conditions was performed before and after lyophilisation confirming enzymatic stability with a specific activity of 5.5 U/mg of protein. Rapidase® was used directly from the commercial

212 packaging with no further treatment. The specific β-glucosidase activity of the commercial
213 preparation was calculated as 0.16 U/mg of protein.

214 *3.2 BglA preliminary assessment in media mimicking wine conditions*

215 As a first assessment of the suitability of *BglA*, a general characterization of the enzyme activity and
216 stability under different conditions and in buffers mimicking different stages of wine processing was
217 carried out. The enzyme retained 32 % and 90 % of activity when tested in the presence of 5 % (w/v)
218 glucose and fructose respectively, and 80 % in the presence of 10 % (v/v) ethanol (See section 2 of
219 the supplementary information). However, prolonged incubation of the enzyme in the different
220 conditions, did not affect the enzyme structural integrity and a 100 % recovered activity was observed
221 in all cases (See section 3 of the supplementary information).

222 *3.3 Enzymatic stability*

223 Stability assays of *BglA* and Rapidase® were also carried out in more complex media; two model
224 wine systems (MW1 and MW2) and a model juice (MJ) were selected to mimic operational
225 conditions. In addition, two real wine matrices (a white, WW, and a red, RW) and a white grape juice
226 (WJ) were included in the screening to assess the performance and longevity of both enzymes in real
227 complex matrices. The appropriate amount of lyophilised enzyme was dissolved in the different
228 systems and incubated for varying periods of time at 22 ºC. An activity test was performed at suitable
229 intervals (1 h, 3 h, 24 h, and 120 h) to assess how the chemical conditions of the matrix (pH, ethanol
230 and sugars) affect the stability of the enzymes.

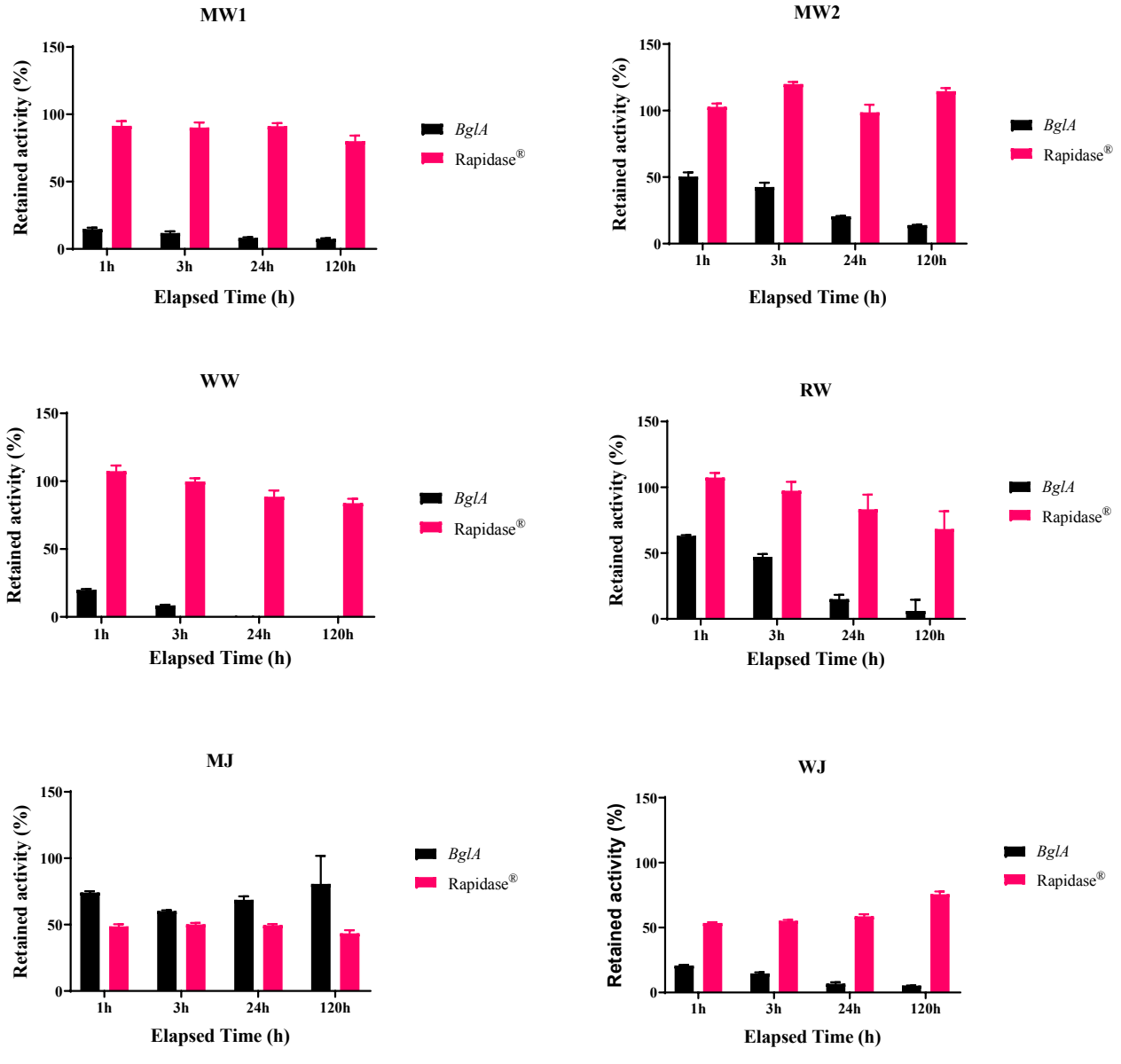


Figure 2. *BglA* and Rapidase® stability assays in Model wine 1 (MW1), Model wine 2 (MW2), White wine (WW), Red wine (RW), Model juice (MJ) and White grape juice (WJ) incubated at 22 ºC during 1 h, 3 h, 24 h and 120 h. Each data point is an average of 3 measurements.

As shown in Figure 2 commercial Rapidase® shows a better stability when incubated in MW1 (10 % ethanol, no sugar, pH 3.5), MW2 (10 % ethanol, 12 g/L glu+fru, pH 3.5), WW (12.2 % ethanol, 4.9 g/L glu+fru, pH 3.35) and RW (13.9 % ethanol, 5.8 g/L glu+fru, pH 3.66), retaining over 60 % activity after 5 days of incubation. *BglA* retains 7 % of activity after 5 days incubation in MW1 and no activity after 24 h in WW. However, after 5 days incubation in MW2, *BglA* shows a retained activity of 14 %, a two-fold increase with respect to MW1. As the sugar content is the only difference between

244 these two model matrices, it appears that fructose and glucose have a protective effect towards *BglA*
245 stability. The stability of *BglA* improves when incubated in RW, retaining 15 % of activity, which is
246 linked to the difference in pH between the two systems; 3.35 for WW (white) and 3.66 for RW (red).
247 On the contrary, Rapidase® shows the opposite behaviour with a 15 % drop in activity when incubated
248 for 5 days in RW in comparison with WW and this could be caused by the higher glucose and fructose
249 content in the red wine (5.8 g/L for the red wine and 4.9 g/L for the white wine) which negatively
250 impacts the stability of the commercial preparation.

251 However, in the model juice MJ (no ethanol, 200 g/L of glu+fruc, pH of 3.7) *BglA* is considerably
252 more stable, retaining around 45% of activity after 120 h incubation. In the same matrix, Rapidase®
253 stability suffers in comparison with its performance in real and model wines, where the pH and the
254 sugar content are significantly lower, and it compares poorly with *BglA*. In white grape juice (WJ),
255 Rapidase® outperforms *BglA*. WJ has less sugar content and lower pH than MJ which clearly impacts
256 *BglA* stability.

257 *3.4 Analytical determination of volatiles released upon enzymatic hydrolysis of glucosides*

258 The hydrolytic capacity of *BglA* and Rapidase® was evaluated with geranyl and guaiacyl glucoside
259 by measuring the release of the free volatiles in the gas phase with SPME-GCMS. To keep the
260 assessment consistent with the stability tests, the catalytic efficiency was also assessed in model
261 systems and real wines as opposed to simpler buffer solutions. The recommended dosage of
262 Rapidase® for white wines is 1 mg of lyophilised powder per hectolitre of wine, and for red wines 2
263 mg/hl. However, *BglA* has been used as a purified preparation in all the assays to better assess its
264 performance. To have consistency among all systems, the effective enzyme quantity has been
265 determined by Bio-Rad protein assay, and the powders weighed to achieve 0.01 mg of protein per
266 mL of matrix in all tests.

267 *3.4.1 Release of volatiles from glucosides in model and real wines*

268 Interestingly, despite a lower stability determined for *BglA* (Fig 1), the catalytic efficiency of this
269 enzyme in MW1 equals that of Rapidase® in the release of geraniol with no significative differences
270 (Table 1). The release of guaiacol by *BglA* is, on the other hand, significantly better after 5 days (97
271 %) in comparison with Rapidase® (75 %). The observed drop in the hydrolysed substrate after 8 days
272 incubation is a known artefact due to the rearrangement of the terpenes under acidic conditions
273 (Hampel, Robinson, Johnson, & Ebeler, 2014; Skouroumounis & Sefton, 2000).

274 When the catalytic performance was assessed in MW2, Rapidase® hydrolytic capacity was
275 diminished in comparison with MW1. The difference between MW1 and MW2 is once again the
276 sugar content. It is known that glucose is a common inhibitor for many β-glucosidases (De Giuseppe
277 et al., 2014) and a content of 6 g/L seems to affect the activity of the commercial preparation. The
278 formation of geraniol is complete after 24 h incubation in samples containing *BglA*, however in the
279 case of Rapidase® 5 days are required to reach complete hydrolysis, compared with 24 h required in
280 MW1. The guaiacol formed in samples containing *BglA* is 62 % after 5 d incubation while with
281 Rapidase® the release of guaiacol after the same incubation period is 6 times lower (10 %). The
282 results are in line with those obtained in the stability assays. The performance of Rapidase® is affected
283 by sugars; probably glucose is causing inhibition of the enzyme. On the other hand, *BglA* tolerates
284 very well high sugar contents.

MW1

Substrate	Enzyme	Time ***[a]	Geraniol released (µg)	% Hydrolysis
Geranyl glucoside	*BglA*	24h	2.33 ± 0.09	96
		5d	2.51 ± 0.11	≥ 99
		8d	2.19 ± 0.05	90
	Rapidase®	24h	2.42 ± 0.03	99
		5d	2.65 ± 0.15	≥ 99
		8d	2.52 ± 0.11	≥ 99
Substrate	**Enzyme *[b]**	**Time ****[b]**	**Guaiaciol released (µg)**	**% Hydrolysis**
Guaiacyl glucoside	*BglA*	24h	1.57 ± 0.07	72
		5d	2.12 ± 0.010	97
		8d	1.20 ± 0.27	55
	Rapidase®	24h	0.64 ± 0.04	29
		5d	1.64 ± 0.12	75

			8d	1.39 ± 0.07	64
MW2	**Substrate**	**Enzyme ***[a]**	**Time ****[a]**	**Geraniol released (µg)**	**% Hydrolysis**
	Geranyl glucoside	*BglA*	24h	2.49 ± 0.08	≥ 99
			5d	2.79 ± 0.07	≥ 99
		Rapidase®	24h	1.33 ± 0.10	55
			5d	2.70 ± 0.10	≥ 99
	Substrate	**Enzyme ****[b]**	**Time ****[b]**	**Guaiacol released (µg)**	**% Hydrolysis**
	Guaiacyl glucoside	*BglA*	24h	0.77 ± 0.04	35
			5d	1.36 ± 0.03	62
		Rapidase®	24h	0.04 ± 0.00	2
			5d	0.23 ± 0.02	10

Table 1. *BglA* and Rapidase® release of geraniol and guaiacol over 24 h, 5 d and 8 d in Model Wine 1 and over 24 h and 5 d in Model Wine 2. ****$\rho \leq 0.0001$; ***$\rho \leq 0.001$; *$\rho < 0.05$. a=geraniol, b=guaiacol.

In comparison with model wines, real wines constitute a highly complex matrix. Without a doubt, underpinning the specific element which either inhibits or destabilises an enzyme is challenging. Potentially, any physical and chemical characteristic of wine is at play: interactions with other molecules, inhibition by sulphur dioxide, rearrangements between components, low pH, sugar content, phenolic glycosides, etc. (Plank et al., 1993). In all cases, hydrolysis was slower and that is reflected in the results.

Rapidase® shows improved activity in WW (Table 2), while the hydrolytic capacity of *BglA* is very limited. On the other hand, after 5 days incubation in RW, *BglA* releases over 30 % geraniol and over 3 % guaiacol. This improvement of the performance of *BglA* in red wine is probably related to a 0.31 pH units difference and 0.9 g/L sugars between white wine and red wine.

WW	**Substrate**	**Enzyme ****[a]**	**Time**	**Geraniol released (µg)**	**% Hydrolysis**
	Geranyl glucoside	BglA	5d	0.01 ± 0.01	0
		Rapidase®	5d	1.94 ± 0.02	80
	Substrate	**Enzyme ***[b]**	**Time**	**Guaiacol released (µg)**	**% Hydrolysis**
	Guaiacyl glucoside	BglA	5d	0.00	0
		Rapidase®	5d	0.24 ± 0.00	11
RW	**Substrate**	**Enzyme **[a]**	**Time**	**Geraniol released (µg)**	**% Hydrolysis**
	Geranyl glucoside	BglA	5d	0.75 ± 0.06	31
		Rapidase®	5d	2.00 ± 0.07	82
	Substrate	**Enzyme *[b]**	**Time**	**Guaiacol released (µg)**	**% Hydrolysis**

Guaiacyl glucoside	BglA	5d	0.07 ± 0.00	3
	Rapidase®	5d	0.23 ± 0.03	11

Table 2. *BglA* and Rapidase® release of geraniol and guaiacol over 5d in White Wine (WW) and Red Wine (RW). ****ρ ≤ 0.0001; ***ρ ≤ 0.001; **ρ ≤ = 0.01; *ρ < 0.05. a=geraniol, b=guaiacol.

3.4.2 Glycosides release in model and real juice

Results in model juice (MJ) (Table 3) highlight an outstanding performance of *BglA* in comparison with the commercial preparation. While Rapidase® hydrolysis capacity is below 6 % for both compounds, the percentage of glycosides hydrolysed by *BglA* is over 60 % for geraniol and over 25 % for guaiacol after 5 days incubation, reaching 45 % after 8 days.

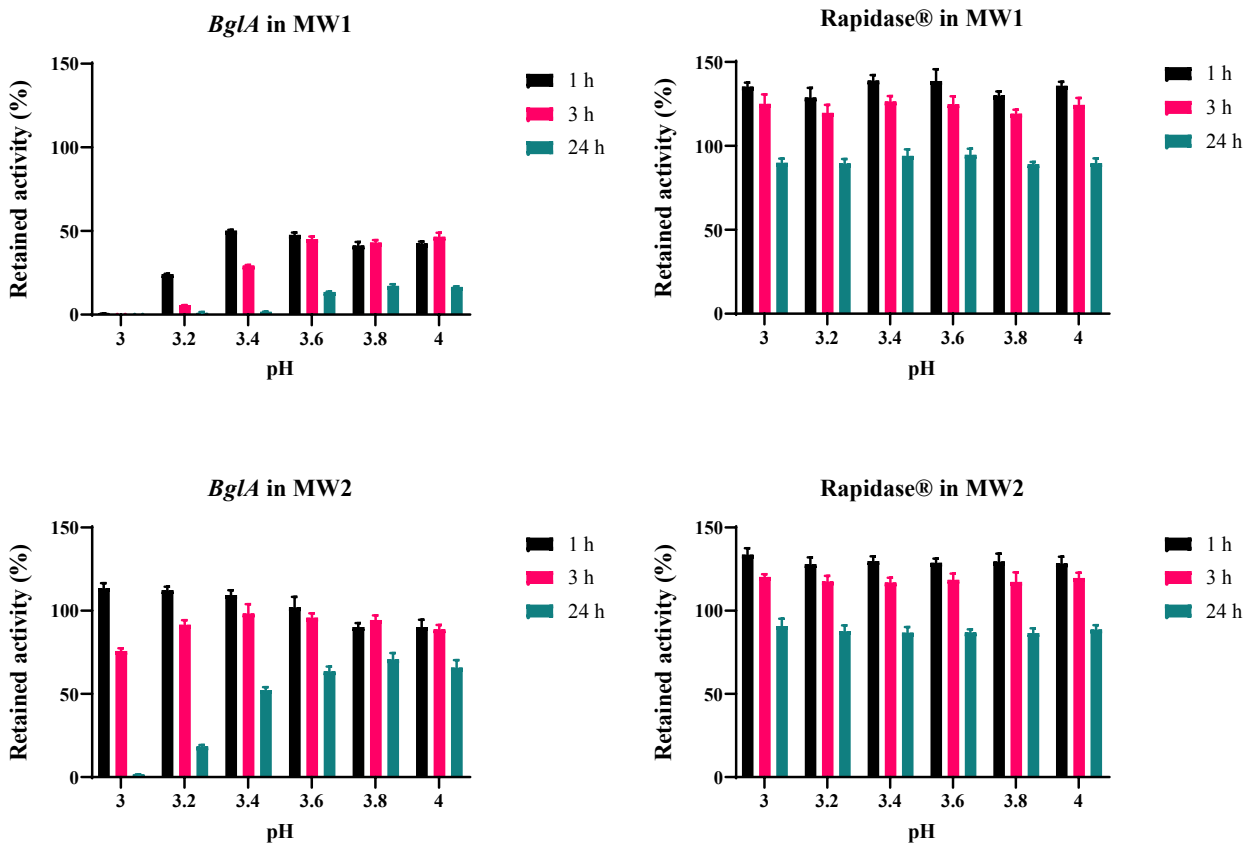
In the case of grape juice (WJ), after 5 days incubation *BglA* continues to show significantly better hydrolysis percentage for geraniol: 10 % against 6 % of Rapidase®. The amount of guaiacol liberated by *BglA* is also slightlyhigher (2 %) than the one released by Rapidase® (1 %).

MJ	**Substrate**	**Enzyme ****[a]**	**Time **[a]**	**Geraniol released (µg)**	**% Hydrolysis**
	Geranyl glucoside	*BglA*	24h	0.94 ± 0.04	39
			5d	1.54 ± 0.04	63
			8d	1.15 ± 0.21	47
		Rapidase®	24h	0.03 ± 0.01	1
			5d	0.07 ± 0.02	3
			8d	0.08 ± 0.02	3
	Substrate	**Enzyme ****[b]**	**Time ****[b]**	**Guaiacol released (µg)**	**% Hydrolysis**
	Guaiacyl glucoside	*BglA*	24h	0.00	0
			5d	0.55 ± 0.11	25
			8d	0.98 ± 0.01	45
		Rapidase®	24h	0.00	0
			5d	0.04 ± 0.00	2
			8d	0.12 ± 0.04	5
WJ	**Substrate**	**Enzyme *[a]**	**Time**	**Geraniol released (µg)**	**% Hydrolysis**
	Geranyl glucoside	*BglA*	5d	0.26 ± 0.01	10
		Rapidase®	5d	0.14 ± 0.01	6
	Substrate	**Enzyme**	**Time**	**Guaiacol released (µg)**	**% Hydrolysis**
	Guaiacyl glucoside	*BglA*	5d	0.03 ± 0.01	2
		Rapidase®	5d	0.02 ± 0.01	1

Table 3. *BglA* and Rapidase® release of geraniol and guaiacol over 24 h, 5 d and 8 d in Model juice (MJ) and over 5 d in real White Juice (WJ). ****ρ ≤ 0.0001; ***ρ ≤ 0.001; **ρ ≤ = 0.01, *ρ < 0.05. a=geraniol, b=guaiacol.

3.5 Detailed pH stability assay in MW1, MW2 and MJ

The results above show that *BglA* loses stability between pH 3 and 4. To further narrow the pH fork causing it, a more accurate stability assay of *BglA* and Rapidase® was carried out with 0.2 pH intervals between pH 3 and 4 in MW1, MW2 and MJ at 22 ºC. Retained activity was measured after 1 h, 3 h, 24 h and 120 h, same intervals as in the enzyme stability experiment in different matrices summarised in Figure 2. Unfortunately, measures after 120 h incubation were no longer reliable, probably due to sample concentration by water loss (results not shown).



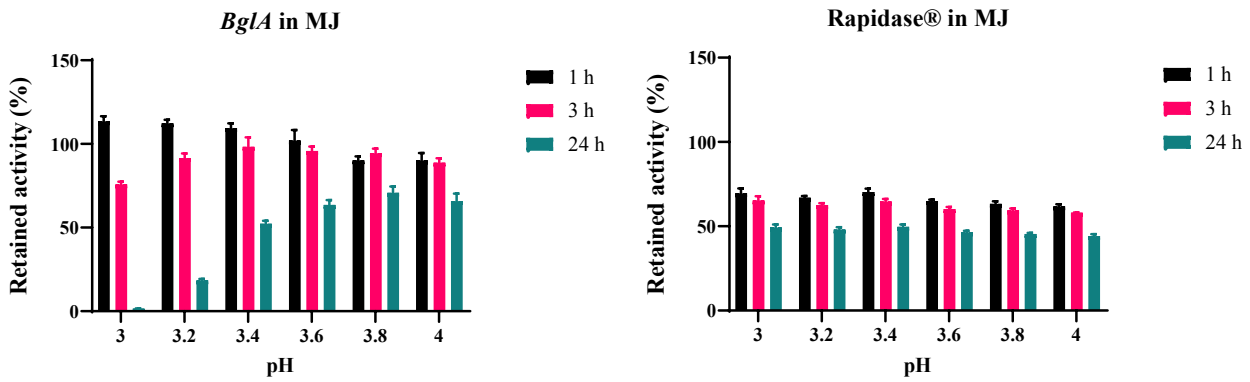


Figure 3. BglA and Rapidase® pH stability assays in Model wine 1 (MW1), Model wine 2 (MW2) incubated at 22 ºC during 1 h, 3 h and 24 h. Each data set is an average of 3 measurements.

BglA loses virtually all activity within 24h of incubation in MW1 and MW2 at a pH lower than 3.6. However, in MJ at pH 3.2, the enzyme still retains 20 % of its activity after the same incubation time. The experiments clearly show that the more sugar the matrix contains, the higher activity *BglA* retains, at all pHs. Rapidase® is clearly independent on pH and the preparation is equally stable between 3 and 4, however, the sugar content present in MJ reduces its activity by almost 50 % very rapidly. These results confirm once again the suitability of *BglA* for matrices with high content of sugars, for example during the maceration or other early stages of the winemaking, previous to the fermentation. Certainly, *BglA* displays great potential for its application in juices. In this work only grape juice has been tested but the results in model juice suggest that any other fruit juice would be a suitable matrix for *BglA*, especially those having a pH over 3.5, like some apple, orange or lemon juices (Yan et al., 2018).

Finally, it is worth to highlight that amounts of freeze-dried protein (mg), and not specific activities (U/mg) have been compared in this study. Due to the lower specific activity of Rapidase® under the same standard conditions, higher amount of freeze-dried preparation of Rapidase® would be required to achieve the same results as *BglA*. Clearly Rapidase® is stable, at least at low sugar content, and when the results are normalised per U of activity its performance is higher, however, from an industrial cost-effective point of view *BglA*, offers both as a crude preparation and in its purified form,

343 13-fold and 34-fold higher activity than the commercial preparation, which results in less quantity of
344 catalyst needed during the wine-making process. In addition, the use of a purified catalyst eliminates
345 the risk of side activities which are always possible in crude preparations and may limit in fact the
346 quantity that can be added to the fermentation process (Sieiro, Villa, Da Silva, García-Fraga, &
347 Vilanova, 2014)

348 **4 Conclusions**

349 β-Glucosidases are used in the wine industry to enhance the aroma of wines and have been proposed
350 to remediate smoke taint defects. The hydrolytic capacity of *BglA* for geraniol glucoside and guaiacol
351 glucoside was significantly better than the commercial preparation in all the tested matrices with high
352 sugar content, where the performance of Rapidase® decreases considerably. In fact, *BglA* high activity
353 in the presence of glucose, outperforms also other reported fungal β-glucosidase such as the one *W.*
354 *anomalus*, which retains only 25 % of activity in the presence of 4 % (w/v) glucose (Sabel et al.,
355 2014), or that from a *A. niger* which retains 64 % of activity when 1 g/L glucose (0.1 % w/v) is added
356 to the reaction but only 2 % when 100 g/L glucose (10 % w/v) is used (Martino et al., 2000). *BglA* is
357 also stable and active in the presence of ethanol as it can be observed from the results in model wines.
358 On the other hand, the activity of *BglA* is very pH dependent and in matrices with a pH below 3.5,
359 like real white wine, the enzyme is not able of hydrolysing glycosides. Future work on enzyme
360 immobilization will be carried out to compare the enzymatic stability at low pH and try to improve
361 it.

362 Great tolerance to sugar content along with improved performance over a broad pH range makes *BglA*
363 an excellent candidate for aroma amelioration and mitigation of smoke taint in grape juices and wines,
364 especially during the early stages of the winemaking process when the sugar content and the pH range
365 is higher than in fermented wines. Future work will also include testing the enzyme in other model
366 and real wines as well as a sensory evaluation of treated wines.

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6 Abbreviations used

ANOVA, analysis of variance; CIS, cooled inlet system; DVB/CAR/PDMS divinylbenzene/carboxen/polydimethylsiloxane, *E.coli*, *Escherichia coli*; GC, gas chromatography; g, gram; hl, hectolitre; h, hours; IPTG, Isopropil-β-D-1-tiogalactopyranoside; MW1, model wine 1; MW2, model wine 2; MJ, model juice; MPS, multipurpose sampler LB, Luria-Bertani; multipurpose sampler; MS, mass spectrometry; µg, microgram; OD, optical density; *p*NPG, paranitrophenol-β-D-Glucopyranoside; RW, red wine; rpm, revolutions per minute; s, seconds; SIM, selected ion monitoring; SPME-GCMS, solid-phase microextraction, gas chromatography mass spectrometry; VP, volatile phenols; v/v, volume volume; w/v, weight per volume; WJ, white juice; WW, white wine.

7 Conflict of interest

The authors declare no conflict of interest in publishing this work.

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