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1 **Antioxidant activity from inactivated yeast: expanding the knowledge**
2 **about the glutathione-related oxidative stability of wine**

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19
20 **Abstract**

21 Maintaining wine oxidative stability during barrel ageing and shelf life storage remains a challenge.
22 This study evaluated the antioxidant activities of soluble extracts from seven enological yeast
23 derivatives (YDs) with increased glutathione (GSH) enrichment. YDs enriched in GSH appeared
24 on average 3.3 times more efficient at quenching radical species than YDs not enriched in GSH.
25 The lack of correlation (Spearman correlation $\rho = 0.46$) between the GSH concentration released

26 from YDs and their radical scavenging activity shed light on other non-GSH compounds present.
27 After 4-methyl-1,2-benzoquinone derivatization, UHPLC–Q-ToF MS analyses specifically
28 identified 52 nucleophiles potentially representing an extensive molecular nucleophilic fingerprint
29 of YDs. The comparative analysis of YD chemical oxidation conditions revealed that the
30 nucleophilic molecular fingerprint of the YD was strongly correlated to its antiradical activity. The
31 proposed strategy shows that nucleophiles co-accumulated with GSH during the enrichment of
32 YDs are responsible for their antioxidant activities.

33

34 **Keywords**

35 Oxidative stability, glutathione, nucleophile, wine, Chardonnay

36

37 **Chemical compounds studied in this article**

38 4-Methylcatechol (PubChem CID: 9958); Hydrogen sulfide (PubChem CID: 402); Acetaldehyde
39 (PubChem CID: 177); Sulfite (PubChem CID: 1099); Hexanal (PubChem CID: 6184); Cysteine
40 (PubChem CID: 5862); Isopentylacetamide (PubChem CID: 263768); Cysteinyl-glycine
41 (PubChem CID: 439498); Hydroxydecanoic acid (PubChem CID: 21488); Glutamyl-cysteine
42 (PubChem CID: 10171468); Glutathione (PubChem CID: 124886)

43

44 **1. Introduction**

45 Early oxidation by nonenzymatic reactions could affect wine quality and thus its economic
46 value. The natural occurrence of transition metals in wine is thought to initiate metal-catalyzed
47 reduction of oxygen, leading to generate hydroperoxyl radicals, which are highly reactive radical
48 oxygen species (ROS), and polyphenol-derived quinones (Danilewicz, 2003). The hydroperoxyl
49 radical reacts quickly and non-selectively with ethanol in wine to yield 85% of 1-hydroxyethyl
50 radical (Elias et al., 2009). The latter is then involved in further chemical reactions with main wine

51 compounds, such as phenols or thiols, resulting in color browning and varietal aroma loss, which
52 are key attributes of wine organoleptic quality, in particular for white wines (Kreitman et al., 2013;
53 Li et al., 2008; Nikolantonaki & Waterhouse, 2012). The genuine antioxidant composition of the
54 wine (phenolic compounds, sulfhydryl compounds, organic acids) regulates the oxidation rate and
55 thus the shelf-life of the wine (Kontogeorgos & Roussis, 2014). In order to preserve wine longer,
56 sulfur dioxide (SO₂) is one of the most versatile and efficient wine antioxidants used to prevent
57 early oxidation. However, intolerances caused by SO₂ derivatives have led to the reduction of its
58 concentration in wines. In a competitive global winemaking market strategy, it is crucial to reduce
59 or even eliminate the use of SO₂ as a preservative and to search for new healthier and safer
60 strategies.

61 Yeast derivatives (YDs) applied biotechnology was proposed a decade ago, as a new strategy to
62 control wine oxidation during bottle storage through oxygen consumption and release of
63 antioxidants (Comuzzo et al., 2015; Pozo-Bayon et al., 2009). Indeed, YDs refer to a class or
64 fraction of yeasts produced on an industrial scale as additives (Pozo-Bayon et al., 2009). Depending
65 on the industrial process, YDs can be found under the form of inactivated yeast, yeast autolysate,
66 yeast protein extract, yeast cell wall and yeast mannoprotein (Comuzzo et al., 2012; Pozo-Bayon
67 et al., 2009).

68 Amongst the numbers of compounds released by YDs in wine, glutathione (GSH) receives most
69 of the scientific attention (Bahut et al., 2019; Kritzinger et al., 2013). This tripeptide containing a
70 cysteine residue is well known to be present naturally in grapes, wine and yeasts. The reductive
71 property supported by the free sulfhydryl enables it to have various beneficial effects during wine
72 aging. GSH has the ability to form colorless products which delay the browning of model white
73 wine under accelerated oxidative conditions (Sonni et al., 2011). In addition, GSH exhibited a
74 protective effect on aromas during aging, notably volatiles esters and terpenes (Papadopoulou &
75 Roussis, 2008) and volatile thiols, and also reduced atypical flavors (Dubourdiou & Lavigne,

76 2004). In parallel, inactivated yeast rich in GSH showed stabilization of wine varietal aromas, such
77 as volatile thiols and terpenes (Gabrielli et al., 2017). Interestingly this study showed that pure
78 GSH at the same concentration as the one released by inactivated yeast had a lower impact, notably
79 on volatile thiols preservation. The combination of GSH with wine antioxidants (phenolic
80 compounds and sulfites at different doses) has shown a positive impact on volatile compounds in
81 long-term wine storage when compared with the use of sulfites alone (Roussis et al., 2013). These
82 results allowed us to hypothesize that the complex chemical composition brought into the must
83 during fermentation by YDs could enhance the formation and/or the stabilization potential of wine
84 aroma at the end of the fermentation.

85 According to a recent study on Chardonnay aged wines, Fourier-transform ion cyclotron
86 resonance mass spectrometry (FTICR-MS) based metabolomics along with multivariate statistical
87 analyses provided evidence that the GSH efficiency against oxidation during bottle aging is
88 dependent on the wine's global antioxidant metabolome, including in particular N- and S-
89 containing compounds like amino acids, aromatic compounds and peptides. These compounds
90 possess a strong nucleophilic character and their reactivity with wine electrophiles, such as
91 oxidized polyphenols, suggests the formation of stable adducts possessing lower oxidative
92 potential (Nikolantonaki et al., 2018). YDs rich in GSH are thus gaining interest since they are a
93 natural way for winemakers to increase the concentration of reduced GSH during winemaking and
94 pre-bottling without direct addition of GSH, which is not yet allowed by European food additives
95 regulations.

96 However, many of these studies about the impact of YDs on wine stability agree to highlight
97 the combined effect of GSH with other compounds released by YDs. Indeed, the metabolic changes
98 related to GSH accumulation in yeast can subsequently impact the diversity of metabolites released
99 by YDs (Bahut et al., 2019). The objective of the present study was to characterize the antiradical
100 effect of different YD products and to give insights into their chemical composition. This work

101 constitutes a primary approach in understanding the action mechanisms of YDs and in establishing
102 better criteria for their use in winemaking. Essentially, no study has shown a clear relationship
103 between the diversity of compounds released by YDs and the potential oxidative stability of wine
104 or other beverages. Our study is dedicated to exploring the stabilization potential properties of
105 different yeast derivatives with a particular emphasis on the non-GSH soluble molecular fraction
106 released in wine-like acidic medium.

107 **2. Materials and methods**

108 *2.1. Chemicals*

109 The water used in this study was ultrapure water (18.2 M Ω ; Millipore, Germany). Ethanol was
110 purchased from Honeywell (United States); formic acid (MS grade), 2,2-diphenyl-1-picrylhydrazyl
111 (DPPH), citric acid and phosphate dibasic from Sigma-Aldrich (St. Louis, MO). Methanol (MS
112 grade and HPLC grade) and acetonitrile (MS grade) were purchased from Biosolve Chimie
113 (Dieuze, France). FeSO₄·7H₂O (99,5%) was purchased from Carlo Erba (Milan, Italy)

114 *2.2. Sample sets*

115 Seven yeast derivatives produced on a laboratory scale were used for this study, labeled YD1 to
116 YD4 and YD6 to YD8 (**Supplementary Information 1**). Each yeast derivative was mixed at 1 g/L
117 in hydro alcoholic solution (12% (v/v) of ethanol with 0.01% (v/v) of formic acid to reach a pH of
118 3.2) previously deoxygenated by bubbling nitrogen through for 10 min. After an hour of stirring at
119 room temperature and in the dark with a rotary stirrer, samples were centrifuged (15 min at 9,000
120 g at 10 °C) and the supernatants were separated and kept at 4 °C until analysis (Bahut et al., 2019).
121 All samples were prepared freshly and analyzed within 24 h to prevent deterioration.

122 *2.3. DPPH radical scavenging activity*

123 The DPPH assay was performed following the protocol previously described (Romanet et al.,
124 2019). A solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was prepared by mixing 27 mg of
125 DPPH with 1L of 60:40 (v/v) 0.3 M buffer citrate-phosphate:methanol to reach a pH of 3.6. In the

126 absence of oxygen, 3.9 mL of the DPPH solution were mixed with 0.1 mL of sample at different
127 mass ratio YD/DPPH (R_m). After 4-h incubation in darkness, sample absorbance was measured in
128 a UV-Vis spectrometer at 525 nm. Absorbance was normalized with the blank (buffer with 0.1 mL
129 of model wine). Results were expressed as the R_m needed to reduce the initial absorbance by 20%,
130 designated $R_{m20\%}$ and translated into the equivalent YD mass to provide the $R_{m20\%}$ (calculation
131 details in **Supplementary Information 2**).

132 *2.4. Nucleophilic compounds derivatization*

133 The derivatization was performed using an adaptation of the protocol described by
134 Nikolantonaki and collaborators (Nikolantonaki & Waterhouse, 2012; Romanet et al., 2020).
135 Firstly, the freshly prepared quinone, 4-methyl-1,2-benzoquinone (4MeQ), is added to 1 mL of
136 sample in excess concentration (final concentration 4MeQ is 1 mM). After 30 min of reaction at
137 room temperature, 1.5 mM SO_2 is added to reduce the remaining 4MeQ in the sample. The addition
138 of quinone in excess allows all nucleophilic compounds present in the soluble fraction of the yeast
139 derivatives to be derivatized. A second set of analyses was performed by adding limiting amounts
140 of 4MeQ, in order to derivatize compounds with the highest affinity for quinone. Six different
141 limiting concentrations (from 30 μM to 625 μM) were added to samples for 30 min before
142 quenching the reaction by addition of 1.5 mM of SO_2 . Samples were then analyzed by high
143 resolution UHPLC-Q-ToF-MS in positive and negative modes with the protocol described below.

144 *2.5. High resolution UHPLC-Q-ToF MS(/MS) analysis*

145 The separation was performed with an ultra-high-pressure liquid chromatography system
146 (Dionex Ultimate 3000; ThermoFisher) coupled to a MaXis plus MQ ESI-Q-TOF mass
147 spectrometer (Bruker, Bremen, Germany). The non-polar and low polar metabolites were separated
148 through reverse-phase liquid chromatography (RP-LC) by injecting 5 μL in an Acquity BEH C_{18}
149 1.7 μm column, 100 \times 2.1 mm (Waters, Guyancourt, France). Elution was performed at 40 $^{\circ}C$ using
150 (A) acidified water (0.1% (v/v) formic acid) and (B) acetonitrile (0.1% (v/v) formic acid) with the

151 following gradient: isocratic step from 0 to 1.10 min with 5% (v/v) B, then the percentage of B was
152 increased to 95% (v/v) until 6.40 min, held there for 3 min and finally returned to the initial
153 condition in 0.1 min for 5 min of re-equilibration. The flow rate was set to 400 $\mu\text{L min}^{-1}$.
154 Electrospray and mass spectrometer acquisition parameters for positive and negative polarity are
155 summarized in **Supplementary Information 3**. A divert valve was used to inject four times diluted
156 ESI-L Low Concentration Tuning Mix (Agilent, Les Ulis, France) at the beginning of each run,
157 allowing a recalibration of each spectrum. The mass spectrometer was calibrated with undiluted
158 Tuning Mix before batch analysis in enhanced quadratic mode, with less than 0.5 ppm errors after
159 calibration. Acquisitions were done in the m/z 100 to 1500 mass range in positive ionization mode.
160 Quality control was used to guarantee the stability of the UHPLC–Q-ToF MS system before each
161 run. Calibrated ions were restricted to those with S/N better than 30 and an absolute intensity of at
162 least 1000. Before features extraction (couple of m/z values and retention time), the spectral
163 background noise was removed. The extracted features were aligned with an in-house R script with
164 a maximum m/z tolerance of 3 ppm and retention time tolerance of 0.5 min and variables absent
165 from more than 80% of the samples were removed from the analysis.

166 Features fragmentation was performed using the Scheduled Precursors List MS/MS function.
167 The fragmentation was performed at two different collision energies: 20 and 35 eV. Parent ions
168 and fragments were submitted to different databases through the massTRIX interface
169 (<http://masstrix.org>) (Suhre & Schmitt-Kopplin, 2008) and YMDB 2.0 (<http://www.ymdb.ca>) and
170 both compositional (obtained isotopic profile) and structural information were used to annotate
171 compounds with high confidence level.

172 2.6. Chemical oxidation monitoring

173 The monitoring of chemical oxidation reactions was performed by UHPLC–Q-ToF MS analysis
174 in negative mode using analytical conditions as described in *Section 2.5*, with the temperature of
175 the auto-sampler set to 30 °C. Chemical oxidation was initiated by mixing 1 mL of YD soluble

176 fraction at 1 g/L with 50 μ L of 4MeC (20 mM) and 5 μ L of FeSO₄·7H₂O (18 mM). Oxidation
177 reactions were monitored after 5 minutes, and then every 50 minutes up to 40 hours after initiation.
178 Samples were analyzed in triplicate.

179 2.7. Data Analysis

180 All experiments were performed at least in triplicate and if not specified, results were expressed
181 as average \pm standard deviation for the triplicate. The basic data mining and data visualization were
182 performed with R software v. 3.5.1. Nonparametric Kruskal-Wallis rank sum tests and Spearman
183 tests were used for median comparison and correlation estimation, respectively. Curve fitting was
184 performed with OriginPro 2017 (b.9.4.0.220).

185 After alignment, features that were positively correlated (Spearman correlation, $\rho > 0.1$) with
186 the addition of the quinone were extracted. They were considered to be associated with compounds
187 which had reacted with quinones. The nucleophile-quinone derivative is the result of the
188 combination of n *4MeC ($1 \leq n$) with m *nucleophiles ($1 \leq m \leq n+2$) leading to m *Nu + n *4MeC
189 addition products (Ma et al., 2019). Thus, a specific m *Nu + n *4MeC derivative carrying the m/z
190 information of the corresponding free nucleophile and the combination of all the derivatives will
191 be used to further express the nucleophilic fingerprints of the YDs. The SmartFormula tool from
192 the DataAnalysis software (v.4.3, Bruker, Germany) allows attribution of a raw formula based on
193 the detected m/z and the isotopic profile (**Supplementary information 5**). Since sulfites were used
194 to quench the derivatization reaction, nucleophilic addition of x *HSO₃ ($0 \leq x$) on 4MeC moiety can
195 be observed to form the nucleophilic addition product m *Nu + n *4MeC + x *HSO₃. The putative
196 raw formula (and thus the corresponding neutral mass) of the free nucleophiles was calculated by
197 subtracting the raw formula of n *4MeC + x *HSO₃ - $2*(n + x + m - 1)*H$ from the total raw formula
198 of the quinone derivative. The resulting nucleophile neutral formula was submitted to a database
199 search (Metlin and YMDB) for putative annotation.

200

201 3. Results and discussions

202 3.1. Radical scavenging activity

203 The measurement of radical scavenging activity was firstly applied to estimate antioxidant
204 properties of pure compounds or mixtures. The radical scavenging activity of the different YD
205 soluble fractions was measured using the DPPH assay (**Figure 2**), recently adapted for wine-like
206 media (Romanet et al., 2019). DPPH is a stable radical in solution with a difference in absorbance
207 between the radical and the protonated form. The decrease of the absorbance can be related to the
208 protonation of the DPPH. It is thus possible to follow the reduction potential of a compound (or a
209 mixture of compounds) with the amount/volume of sample needed to reduce the initial absorbance
210 to a specific range (**Supplementary Information 2**).

211 **Figure 1** shows the mass needed from different YDs to get a 20% decrease (mass equivalent)
212 of the initial absorbance of the DPPH solution. The results of the DPPH assay allowed the following
213 classification of YDs soluble fractions, going from the highest scavenging activity to the lowest:
214 YD8 > YD7 > YD3 > YD1 > YD4 > YD6 > YD2. All tested YDs could reduce the DPPH radical,
215 with YDs rich in GSH (YD8, YD7 and YD3) having the highest antiradical capacity compared to
216 those without GSH accumulation. This result clearly demonstrated the wide range of potential
217 antioxidant properties among the different YDs, with YD8 exhibiting nearly 10 times higher
218 efficiency than YD2.

219 It is important to mention that only soluble fractions were used in this assay (insoluble fractions
220 were removed by centrifugation). Thus, these results represent only a part of the antioxidant
221 potential of the product (except for YD4, which was totally soluble). Indeed, it is known that
222 insoluble fractions also have antioxidant activity, notably due to sulfur-containing compounds
223 present in cell walls and mannoproteins (Jaehrig et al., 2008). In that assay, insoluble fractions
224 were removed to prevent the adsorption of DPPH on cell walls and consequently reduce the

225 concentration (and absorbance) of the radical, which would lead to an overestimation of the
226 antiradical activity of YDs.

227 YD6 which was obtained from the same yeast strain as YD7 (strain B) but without the process
228 leading to GSH accumulation in the intracellular medium, exhibited a 3.8 times lower antiradical
229 activity than that of YD7. The antiradical capacity of YD soluble fractions was related not only to
230 the GSH enrichment industrial process, but also to the yeast strain used. Thus, YD1 (strain A) and
231 YD2 (strain B), which were produced without GSH enrichment exhibited significantly different
232 antiradical scavenging activities, with YD1 being more efficient than YD2. It is also interesting to
233 note the biological variability apparent between YD6 and YD2 obtained from the same strain (B)
234 and the same procedure but in different batches. YD6 was significantly more efficient than YD2
235 (0.72 mg against 0.92 mg respectively) indicating that the technology used to produce the inactive
236 yeast (inactivation procedure, drying system for example) may influence the final activity of the
237 product.

238 To estimate the impact of the GSH concentration on the DPPH results, it was possible to
239 quantify the concentration of GSH in each YD soluble fraction during the assay (red square in
240 **Figure 1**). However, there was no clear relationship between the concentration of GSH during the
241 assay and the antiradical activity of the YDs (Spearman correlation $\rho = 0.46$, p -value > 0.3) despite
242 the known antiradical activity of GSH. YD2, which showed the lowest antiradical activity, was the
243 YD with the highest concentration of GSH during the assay. In contrast, considering equivalent
244 amounts of GSH released by YD8, YD3 and YD1 samples, we observed significant differences in
245 their global antiradical capacity. Therefore, these results led to the conclusion that the activity of
246 the whole soluble fraction (and not only GSH) must impact the antiradical capacity of the YDs
247 estimated by DPPH assay and hence should better explain the classification among YDs than GSH
248 alone. This result is in agreement with a previous study comparing GSH, yeast autolysates and
249 wine lees, where the yeast autolysate (200 mg/L GSH equivalent) showed a greater impact on

250 DPPH discoloration than pure GSH at 500 mg/L (Comuzzo et al., 2015). The potential of the non-
251 GSH fraction on wine aroma stability has also highlighted (Andújar-Ortiz et al., 2010; Rodríguez-
252 Bencomo et al., 2016). Compounds with reducing property such as cysteine-containing compounds
253 could be more abundant than GSH and thus contribute more than GSH to the pool of reductive
254 compounds (Jaehrig & Rohn, 2007; Roussis et al., 2005). GSH is the most abundant non-
255 proteinaceous thiol in yeast, but the accumulation of low concentrations of other sulfhydryl-
256 containing compounds could greatly impact the global reactivity of the matrix against free radicals,
257 or oxidative species (Rodríguez-Bencomo et al., 2014; Roussis et al., 2010). Therefore, the
258 increasing level of these compounds with the enrichment process could explain the differential
259 activities of YD7 and YD6 (which differ only in the production process) and also the highest
260 antiradical activity of YD3, YD7 and YD8 (Bahut et al., 2019).

261 3.2. *Estimation of molecular nucleophilic fingerprints of YD soluble fractions*

262 To go beyond the DPPH method, which does not provide any molecular information related to
263 the antioxidant activity of YDs, we applied a derivatization procedure proposed by Romanet et al,
264 (2020) with an electrophilic probe specifically designed to mimic oxidants in wine, coupled with
265 mass spectrometry detection. Derivatization procedures for the detection and quantification of
266 specific compounds are commonly used to increase the limit of detection (LOD) of targeted
267 compounds. For wine oxidative stability studies, the 4-methylquinone (4MeQ), obtained by
268 oxidation of 4-methylcatechol (4MeC), has been used as a model compound for oxidized
269 polyphenols due to its electrophilic carbon site which could be subject to nucleophilic addition in
270 wine (Danilewicz, 2003, 2013; Danilewicz & Wallbridge, 2010). In addition to sulfites, GSH and
271 ascorbic acid, other nucleophilic compounds, such as thiols, amines and polyphenols, can also
272 competitively react with quinones to form nucleophilic addition products (Nikolantonaki et al.,
273 2014; Waterhouse & Laurie, 2006).

274 The innovative use of untargeted analysis on derivatized and non-derivatized samples enables
275 the detection of nucleophilic compounds specific for wine relevant antioxidants (Inoue et al., 2013;
276 Romanet et al., 2020). Molecular features (m/z pairs and retention time from UHPLC–Q-ToF-MS
277 analyses in both negative and positive ionization modes) were extracted after addition of increasing
278 amounts of 4MeQ in each YD soluble fractions. The reaction of nucleophiles with quinones
279 resulted in the disappearance of the free nucleophiles and the appearance of new products formed
280 after nucleophilic addition. Spearman correlation tests allowed classification of the compounds
281 either as free nucleophiles ($\rho < 0$) or as their addition reaction products ($\rho > 0$). All Spearman
282 correlation scores are given in **Supplementary Information 4**. The combination of positive and
283 negative ionization modes enabled the detection of 85 compounds significantly impacted by the
284 presence of the 4MeQ and common to at least three tested YDs.

285 The great majority of the 85 detected features were detected in negative mode (63) and only 10
286 features were solely detected in positive mode (the other 12 features were also detected in negative
287 mode), which is in agreement with previous results on the efficiency of the negative ionization
288 mode to detect quinone derivatives (Ma et al., 2019). Features corresponding to nucleophilic
289 addition reaction products ($\rho > 0$) represented the majority of the detected compounds in both
290 positive (73% (16/22)) and negative ionization modes (83% (52/63)). Since the addition reaction
291 products correspond to 4MeC addition on nucleophiles, the diversity of these products is related to
292 the diversity of free nucleophiles, which means that the products of nucleophilic addition are
293 representative of the diversity of nucleophiles present in the solution. The large
294 abundance/diversity of quinone derivatives compared to the few free nucleophiles actually detected
295 illustrates the potential of the proposed derivatization method to improve detection of poorly
296 ionizable free nucleophilic compounds. With respect to these observations, quinone derivatives
297 detected in negative mode were selected as representatives of the nucleophilic fraction
298 (nucleophilic fingerprint, **Figure 2**) of the samples and used for further investigation.

299 These 52 nucleophiles enabled the discrimination of the YDs according to their initial nucleophilic
300 fingerprint and thus their potential antioxidant activity. As previously reported, YD4 appeared
301 chemically significantly different from the others with few nucleophiles (26 detected) at low
302 abundance. Besides YD4, three clusters were clearly defined: YD7-YD8, YD1-YD3 and YD6-
303 YD2. The absolute number of nucleophiles was equivalent (average of 42 ± 2 compounds) between
304 these YDs, but they showed important molecular diversity. Besides the 26 common nucleophiles
305 shared by YD7-YD8, YD1-YD3 and YD6-YD2, 16 compounds were specific to some of these
306 YDs. The chemical proximity between the YD nucleophilic fingerprints is relevant information to
307 attribute similar antioxidant activities to similar samples. In order to estimate this parameter, the
308 principal components analysis of the nucleophilic fingerprint features was performed to reduce the
309 number of dimensions of the data and get an overview of the sample's distance (**Figure 3**). In that
310 case, the nucleophilic fingerprint allowed the hierarchization of YDs consistent with that obtained
311 from the DPPH assay.

312 The DPPH assay revealed the failure of the GSH concentration to explain the scavenging
313 activity of YDs soluble fractions. However, the derivatization procedure highlighted the potential
314 of nucleophiles that were unconsidered until now, to better characterize the antiradical activity of
315 YD soluble fractions. This study showed the major importance of the non-targeted approach to
316 consider the global nucleophilic fingerprint for a better assessment of the antioxidant potential of
317 YD soluble fractions. In the present case, the correlation circle plots revealed the features which
318 specifically discriminate the different YDs and pointed out the most relevant (out of the inner circle
319 representing a correlation of 0.8).

320 In addition to the nucleophilic fingerprint, the high resolution of the UHPLC-Q-ToF-MS has
321 been used to putatively annotate the nucleophiles. Based on the derivatization method, the
322 nucleophilic addition reaction could occur between one or several nucleophiles with one or two
323 electrophilic sites of the 4MeQ (Ma et al., 2019; Nikolantonaki et al., 2012, 2014; Nikolantonaki

324 & Waterhouse, 2012; Romanet et al., 2020). **Table 1** summarizes the putative annotations of the
 325 quinone derivatives detected in negative ionization mode, which were positively correlated to the
 326 addition of 4MeQ.

327
 328 **Table 1:** Relevant nucleophiles detected in negative ionization mode and their putative annotation based on
 329 mass precision and isotopic profiles. The isotopic profile and the MS² profile are available in
 330 **Supplementary Information 5** for compounds detected with relative intensities higher than 1000. Mono
 331 and di-deprotonated free forms of 4-methylcatechol C₇H₇O₂ (*m/z* = 123.0452) and C₇H₆O₂ (*m/z* = 122.0375)
 332 respectively as well as the auto-polymerization products C₁₄H₁₃O₄ (*m/z* = 245.0819) and C₁₄H₁₃O₄ (*m/z* =
 333 247.0977) were excluded from the data mining.

<i>m/z</i> _{experimental}	RT	Ion putative formula	Δppm	Adduct	[M]	[M] neutral Formula	YMDB ID	Annotation*
155.0171	1.9	C ₇ H ₇ O ₂ S	-0.80	[(M+4MeC-2H)-H] ⁻	33.9877	H ₂ S	YMDB00653	hydrogen sulfide
165.0557	3.2	C ₉ H ₉ O ₃	-0.11	[(M+4MeC-2H)-H] ⁻	44.0262	C ₂ H ₄ O	YMDB00022	acetaldehyde
203.002	1.3	C ₇ H ₇ O ₅ S	0.16	[(M+4MeC-2H)-H] ⁻	81.9725	H ₂ SO ₃	YMDB00114	sulfite*
203.0021	2.9	C ₇ H ₇ O ₅ S	0.65	[(M+4MeC-2H)-H] ⁻	81.9725	H ₂ SO ₃	YMDB00114	sulfite
215.0675	2.6	C ₈ H ₁₁ N ₂ O ₅	0.72	[(M+4MeC-2H)-H] ⁻	94.0378	CH ₆ N ₂ O ₃	-	unknown
221.1183	4.5	C ₁₃ H ₁₇ O ₃	-0.08	[(M+4MeC-2H)-H] ⁻	100.0888	C ₆ H ₁₂ O	YMDB16016	hexanal*
242.0492	1.9	C ₁₀ H ₁₂ NO ₄ S	-0.21	[(M+4MeC-2H)-H] ⁻	121.0197	C ₃ H ₇ NO ₂ S	YMDB00046	cysteine
250.145	6.3	C ₁₄ H ₂₀ NO ₃	0.53	[(M+4MeC-2H)-H] ⁻	129.1154	C ₇ H ₁₅ NO	YMDB16052	isopentylacetamide*
265.148	5.9	C ₁₂ H ₂₅ O ₄ S	0.36	[(M+4MeC-2H)-H] ⁻	142.1028	C ₅ H ₁₈ O ₂ S	-	unknown
272.089	2.5	C ₁₀ H ₁₄ N ₃ O ₆	0.70	[(M+4MeC-2H)-H] ⁻	151.0593	C ₃ H ₉ N ₃ O ₄	-	unknown
283.0395	1.3	C ₁₁ H ₁₁ N ₂ O ₅ S	0.30	[(M+4MeC-2H)-H] ⁻	162.0099	C ₄ H ₆ N ₂ O ₃ S	-	unknown
293.1789	8.0	C ₁₄ H ₂₉ O ₄ S	-1.04	[(M+4MeC-2H)-H] ⁻	172.1497	C ₇ H ₂₄ O ₂ S	-	unknown
299.0707	2.0	C ₁₂ H ₁₅ N ₂ O ₅ S	-0.05	[(M+4MeC-2H)-H] ⁻	178.0412	C ₅ H ₁₀ N ₂ O ₃ S	YMDB00690	Cys-Gly
300.0092	3.2	C ₁₃ H ₆ N ₃ O ₄ S	2.50	[(M+4MeC-2H)-H] ⁻	178.9789	C ₆ HN ₃ O ₂ S	-	unknown*
309.1709	5.1	C ₁₇ H ₂₅ O ₅	0.49	[(M+4MeC-2H)-H] ⁻	188.1412	C ₁₀ H ₂₀ O ₃	YMDB16207	hydroxydecanoic acid
309.1741	6.7	C ₁₄ H ₂₉ O ₅ S	-0.06	[(M+4MeC-2H)-H] ⁻	216.1508	C ₇ H ₂₄ N ₂ O ₃ S	-	unknown
325.0352	0.7	C ₉ H ₁₃ N ₂ O ₉ S	1.46	[(M+4MeC+H ₂ SO ₃ -4H)-H] ⁻	124.0484	C ₂ H ₈ N ₂ O ₄	-	unknown
353.2002	7.4	C ₁₆ H ₃₃ O ₆ S	-0.38	[(M+4MeC-2H)-H] ⁻	232.1708	C ₉ H ₂₈ O ₄ S	-	unknown
353.2485	7.1	C ₁₆ H ₃₇ N ₂ O ₄ S	1.55	[(M+4MeC-2H)-H] ⁻	232.2184	C ₉ H ₃₂ N ₂ O ₂ S	-	unknown

355.1581	7.4	C ₁₈ H ₂₇ O ₅ S	-1.04	[(M+4MeC-2H)-H] ⁻	234.1290	C ₁₁ H ₂₂ O ₃ S	-	unknown*
371.0922	2.6	C ₁₅ H ₁₉ N ₂ O ₇ S	0.95	[(M+4MeC-2H)-H] ⁻	250.0623	C ₈ H ₁₄ N ₂ O ₅ S	YMDB00252	Glu-Cys*
386.0339	0.7	C ₁₀ H ₁₆ N ₃ O ₉ S ₂	1.44	[(M+4MeC+H ₂ SO ₃ -4H)-H] ⁻	185.0470	C ₃ H ₁₁ N ₃ O ₄ S		unknown
398.2336	7.2	C ₂₄ H ₃₂ NO ₄	-0.21	[(M+4MeC-2H)-H] ⁻	277.2042	C ₁₇ H ₂₇ NO ₂	-	unknown
409.3111	7.8	C ₂₈ H ₄₁ O ₂	-0.25	[(M+4MeC-2H)-H] ⁻	288.2777	C ₂₁ H ₃₆	-	unknown
423.0821	0.7	C ₁₃ H ₁₉ N ₄ O ₁₀ S	-1.51	[(M+4MeC+H ₂ SO ₃ -4H)-H] ⁻	302.0505	C ₆ H ₁₄ N ₄ O ₅	-	unknown*
426.0979	2.5	C ₁₈ H ₁₆ N ₇ O ₄ S	-2.57	[(M+4MeC-2H)-H] ⁻	305.0695	C ₁₁ H ₁₁ N ₇ O ₂ S	-	unknown
428.1136	2.5	C ₁₇ H ₂₂ N ₃ O ₈ S	0.68	[(M+4MeC-2H)-H] ⁻	307.0838	C ₁₀ H ₁₇ N ₃ O ₆ S	YMDB00160	glutathione*
431.1151	2.5	C ₁₀ H ₁₅ N ₁₂ O ₈	2.25	[(M+4MeC-2H)-H] ⁻	310.0846	C ₃ H ₁₀ N ₁₂ O ₆	-	unknown
432.0757	0.7	C ₁₂ H ₂₂ N ₃ O ₁₀ S ₂	1.14	[(M+4MeC+H ₂ SO ₃ -4H)-H] ⁻	231.0889	C ₅ H ₁₇ N ₃ O ₅ S	-	unknown
439.0807	0.7	C ₁₃ H ₁₉ N ₄ O ₁₁ S	-0.74	[(M+4MeC+H ₂ SO ₃ -4H)-H] ⁻	318.0481	C ₃ H ₁₈ N ₄ O ₆ S	-	unknown
451.0488	1.3	C ₁₅ H ₁₉ N ₂ O ₁₀ S ₂	0.31	[(M+4MeC+H ₂ SO ₃ -4H)-H] ⁻	250.0623	C ₈ H ₁₄ N ₂ O ₅ S	YMDB00252	Glu-Cys*
481.0248	2.5	C ₂₃ H ₁₃ O ₁₀ S	2.72	[(M+4MeC+H ₂ SO ₃ -4H)-H] ⁻	290.1154	C ₁₆ H ₁₈ O ₅	-	unknown
502.963	5.4	C ₁₅ H ₁₁ N ₄ O ₁₀ S ₃	-2.54	[(M+4MeC-2H)-H] ⁻	381.9348	C ₈ H ₆ N ₄ O ₈ S ₃	-	unknown
508.0704	1.2	C ₁₇ H ₂₂ N ₃ O ₁₁ S ₂	0.54	[(M+4MeC+H ₂ SO ₃ -4H)-H] ⁻	307.0838	C ₁₀ H ₁₇ O ₆ N ₃ S ₁	YMDB00160	glutathione*
526.0809	2.5	C ₁₈ H ₂₀ N ₇ O ₈ S ₂	-2.14	[(M+4MeC-2H)-H] ⁻	405.0525	C ₁₁ H ₁₅ N ₇ O ₆ S ₂	-	unknown
550.1502	3.4	C ₂₄ H ₂₈ N ₃ O ₁₀ S	0.20	[(M+4MeC+H ₂ SO ₃ -4H)-H] ⁻	349.1638	C ₁₇ H ₂₃ N ₃ O ₅	Metlin_17974	Tyr-Pro-Ala*
572.1318	3.4	C ₂₂ H ₂₂ N ₉ O ₈ S	0.08	[(M+4MeC-2H)-H] ⁻	451.1023	C ₁₅ H ₁₇ N ₉ O ₆ S	-	unknown
676.1594	2.3	C ₂₅ H ₃₄ N ₅ O ₁₃ S ₂	-0.89	[(M+4MeC+H ₂ SO ₃ -4H)-H] ⁻	475.1737	C ₁₈ H ₂₉ N ₅ O ₈ S	200142	Pro-Cys-Gln-Glu
733.1809	2.1	C ₂₇ H ₃₇ N ₆ O ₁₄ S ₂	-0.77	[2(M+4MeC-2H)-H] ⁻	307.0838	C ₁₀ H ₁₇ N ₃ O ₆ S	YMDB00160	glutathione
800.2121	2.6	C ₂₄ H ₃₈ N ₁₁ O ₁₈ S	-0.18	[(M+4MeC-2H)-H] ⁻	679.1827	C ₁₇ H ₃₃ N ₁₁ O ₁₆ S	-	unknown
857.234	2.5	C ₃₄ H ₄₅ N ₆ O ₁₆ S ₂	0.12	2[(M+4MeC-2H)-H] ⁻	858.2418	C ₁₀ H ₁₇ N ₃ O ₆ S	YMDB00160	glutathione*

334 Δ ppm is calculated as: $\frac{m/z_{\text{experimental}} - m/z_{\text{theoretical}}}{m/z_{\text{theoretical}}} \times 10^6$; $m/z_{\text{theoretical}}$ corresponds to the exact m/z of the ion putative formula.

335 *Features for which MS/MS profile is provided in supplementary information 5

336

337

338 Almost 60% (26/44) of the nucleophiles detected were not found in online databases, whatever the
339 combination of 4MeC or HSO₃-4MeC used. Of the 41 attributed elemental formulas, 21 could be
340 putatively assigned to sulfur containing compounds.

341 Within the annotated adducts, eight were related to GSH or GSH precursors such as cysteine
342 (C₃H₇NO₂S, *m/z* = 120.0125) and glutamyl-cysteine (C₈H₁₄N₂O₅S, *m/z* = 249.0551): [(Cysteine-
343 4MeC-2H)-H]⁻ (C₁₀H₁₂NO₄S, *m/z* = 242.0492), [(Cys-Gly+4MeC-2H)-H]⁻ (C₁₂H₁₅N₂O₅S, *m/z* =
344 299.0707), [(Glu-Cys+4MeC-2H)-H]⁻ (C₁₅H₁₉N₂O₇S, *m/z* = 371.0922), [(GSH+4MeC-2H)-H]⁻
345 (C₁₇H₂₂N₃O₈S, *m/z* = 428.1136), [(Glu-Cys+4MeC+H₂SO₃-4H)-H]⁻ (C₁₅H₁₉N₂O₁₀S₂, *m/z* =
346 451.0488), [(GSH+4MeC+H₂SO₃-4H)-H]⁻ (C₁₇H₂₂N₃O₁₁S₂, *m/z* = 508.0704), [2(GSH+4MeC-
347 2H)-H]⁻ (C₂₇H₃₇N₆O₁₄S₂, *m/z* = 733.1809) and 2[(GSH+4MeC-2H)-H]⁻ (C₃₄H₄₅N₆O₁₆S₂, *m/z* =
348 857.234_2.49). This agrees with the nature of the YDs, since three of these products had been
349 produced in order to accumulate GSH (YD3, YD7 and YD8). The high concentration of GSH and
350 its precursors could explain the abundance of adducts containing these specific nucleophiles. This
351 observation was also corroborated by the correlation circle plot in **Figure 3** where the separation
352 between YD3, YD7 and YD8 is strongly correlated ($\rho > 0.8$) to the abundance of [371.0922_2.55]
353 and [733.1809_2.13], which correspond to Glu-Cys and GSH derivatives, respectively, and to a
354 lower extent to [428.1136_2.45] and [857.234_2.49], which also correspond to GSH derivatives.

355 In **Figure 3**, the opposite direction is driven by the abundance of sulfite derivatives
356 [203.0021_2.87] and [203.0020_1.31] in the samples YD2 and YD6. These two compounds
357 correspond to sulfite addition on 4MeC, likely in different C electrophilic sites. The nucleophilic
358 addition of SO₂ is known to be minor in comparison with the reduction of the quinone (11% yield
359 against 89%, respectively) (Nikolantonaki & Waterhouse, 2012). Thus, the abundance of
360 sulfonated 4MeC could provide relative information about the underivatized fraction of the 4MeQ.

361 Besides the GSH and GSH precursor derivatives, few other compounds had been annotated from
362 online databases. Notably two peptides Tyr-Pro-Ala (C₂₄H₂₈N₃O₁₀S, *m/z* = 550.1502) and Pro-Cys-

363 Gln-Glu ($C_{25}H_{34}N_5O_{13}S_2$, $m/z = 676.1594$), which were strongly correlated with the abundance of
364 glutamyl-cysteine and GSH derivatives (**Figure 3**). The correlation between two compounds could
365 indicate the co-accumulation of these peptides during the GSH accumulation process, or the
366 degradation product coming from specific macromolecules involved in the GSH accumulation
367 process. The nucleophilic property of these peptides highlights the wider effect of the GSH
368 enrichment process on a diversity of other metabolites. The quality of the growing environment,
369 notably nutritious factors, is known to impact the genome expression and thus the metabolome of
370 yeasts (Kresnowati et al., 2006). During the industrial process, this leads to the accumulation of
371 specific metabolites, such as reduced GSH. It was shown that the transient presence of specific
372 nutrients in the yeast culture media can produce yeast with distinct growth and compositional
373 characteristics (Alfajara et al., 1992). Moreover, it was recently shown that the accumulation of
374 GSH in inactivated *Saccharomyces cerevisiae* yeasts is associated with an increased production of
375 multiple cysteine-containing peptides and other sulfur containing compounds. Finally, **Table 1**
376 shows that 13 nucleophiles were formed with compounds which do not contain sulfur. This further
377 indicates the clear potential of chemical families other than thiols to contribute to the antiradical
378 activity of YDs. For example, aldehydes such as acetaldehyde ($C_9H_9O_3$, $m/z = 165.0557$) and
379 hexanal ($C_{13}H_{17}O_3$, $m/z = 221.1183$) had been annotated as potential 4MeQ binders. These results
380 are in accordance with those found previously in wine, where the reaction between aldehydes and
381 polyphenols is a major step in pigments formation (Li et al., 2008; Oliveira et al., 2011; Waterhouse
382 & Laurie, 2006).

383 3.3. *The role of soluble fraction of YDs on chemically initiated oxidation under wine-like* 384 *conditions*

385 If the derivatization procedure provided the total relevant nucleophiles present in the solution,
386 which were able to react with an excess concentration of quinone, it must be considered that the
387 oxidation of catechol into quinone is a gradual process catalyzed by metal transition. During this

388 slow oxidation, distinct nucleophiles do not have the same affinity towards the quinone and thus
389 are submitted to competitive additional reactions (Nikolantonaki et al., 2012, 2014; Nikolantonaki
390 & Waterhouse, 2012). Under chemical oxidation conditions in wine-like medium, the oxidation
391 reaction rate is related to the rate of reactive oxygen species formation *via* the oxidation of
392 polyphenols catalyzed by the presence of metals (Danilewicz et al., 2008; Elias & Waterhouse,
393 2010).

394 In order to investigate the reactivity of YDs under chemical oxidation conditions, YD2, YD3
395 and YD8 soluble fractions, selected for their low, medium and high nucleophilicity (respectively),
396 according to the different clusters shown **Figure 2** and their antiradical properties (**Figure 1**), were
397 submitted to chemical oxidation in the presence of a model polyphenol (4MeC) and of Fe²⁺.
398 Reactions were conducted at 30 °C and monitored over 40 hours, with data collection every 50
399 min, and then processed to identify nucleophiles strongly correlated with oxidation (Spearman
400 correlation, $\rho \geq |0.85|$). For each *m/z*_retention time couple, the peak area was centered on its initial
401 value (time = 5 min) and divided by the standard deviation for behavior comparison on a common
402 scale. **Figure 4** represents the evolution of five features during chemical oxidation, considered as
403 representative for kinetic profiles of all detected features.

404 The production of the oxidant (4MeQ) begins immediately after mixing Fe²⁺ and 4MeC. The
405 rapid increase of GSH-4MeC in the three YDs shows that the 4MeQ was quickly quenched by the
406 GSH which decreased gradually in parallel. After 20 h of oxidation, the free GSH in YD2 is
407 completely depleted while GSH-4MeC reaches a plateau. In contrast, GSH consumption in YD3
408 and YD8 was not complete even after 40 h (**Supplementary Information 6-8**). The observed
409 differences between GSH consumption kinetic rates could be guided by the concentration effect
410 and synergic/antagonistic effects, due to the presence of other compounds competing during its
411 nucleophilic addition reaction with the quinone. Indeed, similar to GSH, the unknown nucleophile
412 C₈H₁₆N₂O₉S₂ (*m/z* = 347.0232) specific to YD3 and YD8, was totally depleted in YD3 but not in

413 YD8 after 30 h. These results highlight the importance of the chemical diversity of the nucleophilic
414 fingerprints of the YDs for the comprehension of their antioxidant capacity.

415 Besides the production of GSH-4MeC, the double addition product GSH-4MeC-GSH
416 (equivalent to $C_{27}H_{37}N_6O_{14}S_2$, $m/z = 733.1805$) was not observed in all samples. It never appeared
417 in YD2 while it appeared with delay in YD3 (after 14 hours) and YD8 (after 10 hours). The
418 production of GSH-4MeC-GSH can be related to (i) a further oxidation of GSH-4MeC derivatives
419 and (ii) a nucleophilic addition of GSH to this specific electrophile. Thus, the higher the
420 concentration of the GSH-4MeC adduct (and the corresponding oxidized form) and that of the
421 remaining free GSH, the quicker the onset of the double adduct production. This agrees with the
422 quicker appearance and evolution of this double adduct for YD8 than for YD3, where the
423 concentration of free GSH was initially higher. The feature [299.0706_1.96] (annotated as [(Gly-
424 Cys+4MeC-2H)-H]⁻) exhibited a particular behavior in this example. In YD2, it quickly increased
425 during the first 13 h, reached a maximum and then decreased until total disappearance 15 h later.
426 This reaction could be explained by the rapid nucleophilic addition of Gly-Cys on 4MeQ until a
427 maximum corresponding to the total derivatization of the Gly-Cys. Then the decrease would be
428 interpreted as a second reaction occurring on the simple adduct, such as a second nucleophile
429 addition on the Gly-Cys-4MeC. It is also interesting to note the highly different kinetic of
430 appearance of the Gly-Cys-4MeC. In YD2, the reaction was fast and led to the total derivatization
431 of Gly-Cys, whereas in YD3 and YD8 the reaction occurred with delay and was slow. The presence
432 of competitive reactions for nucleophilic addition could explain these differences as already
433 reported in the literature (Nikolantonaki et al., 2014). Nucleophilic competition can therefore
434 actively modulate the production rate of specific quinone adducts and thus modulate the final
435 chemistry of the wine.

436 In order to semi-quantify the nucleophilic potential of YDs soluble fractions, the reaction rates
437 of GSH consumption and GSH-4MeC production were calculated directly in their native complex

438 chemical environments. To that purpose, first order kinetic (**Equation 1**) was used to express GSH
439 and GSH-4MeC reaction rates during oxidation:

$$440 \quad A_{(t)} = A_0 + B * e^{-R*t} \quad \text{Equation 1}$$

441 With the following constants:

442 $A_{(t)}$: Area at time t (a.u.)

443 A_0 : Area offset (a.u.)

444 B : Initial Area (a.u.)

445 R : Rate of the reaction (h^{-1})

446 t : time of oxidation (h)

447

448 **Table 2** presents the fitting parameters for the curves of GSH consumption and GSH-4MeC
449 production for YD2, YD3 and YD8, using Equation 1. Extensive results such as fitted curves for
450 all replicates and residuals plots are available in **Supplementary Information 9–11**. The fit with
451 a first order equation matched well with the GSH raw data (adjusted $R^2 > 0.98$ for all replicates).
452 This indicates that the nucleophilic addition of GSH to 4MeQ must be the main reaction leading to
453 the consumption of GSH. In contrast, the production of GSH-4MeC seemed more complex. Since
454 GSH-4MeC-GSH was found in YD3 and YD8, it showed that an equilibrium exists between the
455 GSH-4MeC increase (nucleophilic addition of GSH on 4MeQ) and GSH-4MeC decrease by further
456 nucleophilic addition (for example, nucleophilic addition of GSH on GSH-4MeQ to form GSH-
457 4MeC-GSH present in YD3 and YD8). In the latter reaction, GSH could be replaced by any other
458 nucleophile present in the solution. The apparent first order of the reaction actually hides a complex
459 balance between the appearance of GSH-4MeC and the disappearance of this compound in further
460 reactions. For example, the progressive disappearance of GSH-4MeC is clearly visible in YD2
461 (**Figure 4**) after 20 h. On the basis of these few acknowledged reaction pathways for GSH, we only
462 considered the GSH kinetics for further analysis. The kinetic rate of GSH given by the R parameter
463 in **Equation 1** indirectly estimates the dynamic of reactions occurring in the solution between this
464 compound and its chemical environment.

465 **Table 2:** Parameters of the first order fit (**Equation 1**) and associated half-life time ($t_{1/2}$) and adjusted R^2 .
 466 Different letters represent significant differences after Wilcoxon test, $n = 3$ and p -value < 0.05 .

GSH								
	A_0 (a.u.)		B (a.u.)		R (h^{-1})		$t_{1/2}$ (h)	adj. R^2
YD2	$-9.8 \times 10^3 \pm 4.4 \times 10^3$	a	$5.9 \times 10^5 \pm 2.7 \times 10^4$	a	0.15 ± 0.01	a	4.6	0.98
YD3	$-3.1 \times 10^3 \pm 2.3 \times 10^5$	a	$1.4 \times 10^6 \pm 2.5 \times 10^5$	ab	0.03 ± 0	ab	23.1	0.98
YD8	$-1.5 \times 10^6 \pm 7.8 \times 10^5$	a	$4.0 \times 10^6 \pm 8.2 \times 10^5$	b	0.01 ± 0	b	69.3	0.99

GSH-4MeC								
	A_0 (a.u.)		B (a.u.)		R (h^{-1})		$t_{1/2}$ (h)	adj. R^2
YD2	$2.6 \times 10^6 \pm 3.9 \times 10^4$	a	$-2.1 \times 10^6 \pm 1.1 \times 10^5$	a	0.36 ± 0.01	a	1.9	0.88
YD3	$3.4 \times 10^6 \pm 3.2 \times 10^5$	ab	$-2.3 \times 10^6 \pm 3.4 \times 10^4$	a	0.07 ± 0.02	ab	9.9	0.92
YD8	$4.9 \times 10^6 \pm 1.4 \times 10^5$	b	$-3.7 \times 10^6 \pm 1.7 \times 10^5$	a	0.03 ± 0	b	23.1	0.94

467
 468 Parameters A_0 and B give information about the initial and final area of the compound, while R
 469 is representative of the global rate of the reaction. In order to compare the rates of reaction between
 470 YDs, the half-life constants ($t_{1/2}$, time needed to achieve 50% of the reaction) were calculated as
 471 follows (**Equation 2**):

$$472 \quad t_{1/2} = \frac{\ln(2)}{R} \quad \text{Equation 2}$$

473 YD2 was the only sample where GSH reached total depletion, which gave access to the
 474 experimental $t_{1/2}$. The $t_{1/2}$ was estimated to be 4.6 hours (**Equation 2**) with the fit, whereas the
 475 experimental value was 3.2 ± 0.3 hours. The estimation is thus close to the experimental $t_{1/2}$ which
 476 consolidates the validity of first order kinetics to estimate the rate of the reaction. Consumption of
 477 GSH is significantly quicker for YD2 with a $t_{1/2}$ fifteen and five times lower than YD8 and YD3,
 478 respectively.

479 These results suggest that compounds present in YD samples can interfere with the oxidative
 480 chain reaction, normally leading to the reaction of GSH with 4MeQ to produce GSH-4MeC,
 481 through possibly more kinetically favorable reactions. Our study enabled us to appreciate the
 482 influence of these reactions by tracing the kinetics of GSH consumption. GSH can be used as an
 483 indirect marker to estimate the activity of the pool of nucleophiles to preserve GSH itself. Indeed,
 484 the slower kinetic of consumption of GSH (YD8) would be the result of a matrix able to strengthen

485 the action of GSH and thus to preserve this oxidation-sensitive compound. Samples with higher
486 diversity and abundance of nucleophiles (YD3 and YD8) present longer $t_{1/2}$ for consumption of
487 GSH and production of the addition product. In this context, YDs can be ordered from the more
488 nucleophilic to the less nucleophilic: YD8 > YD3 > YD2 based on their $t_{1/2}$. The low abundance of
489 additional nucleophiles in YD2 led to a rapid decrease of GSH until total depletion. YD3 and YD8
490 released much more GSH in solution compared to YD2 and did not reach the plateau after 40 h of
491 oxidation. But interestingly, they exhibited high differences in $t_{1/2}$. Associated with the higher
492 amount of GSH, YD8 exhibited a slower (but not significant at p -value ≤ 0.05) consumption rate
493 than YD3. This shows that independently of the concentration of GSH, the co-accumulated
494 nucleophiles effectively preserve the pool of GSH. This result is in accordance with our previous
495 results showing the positive impact of the GSH accumulation process on the quality and quantity
496 of potential nucleophilic compounds (Bahut et al., 2019).

497 In addition, this nucleophilic order (YD8 > YD3 > YD2) order matches perfectly with the
498 antiradical activity of these samples found with the DPPH assay. The DPPH assay demonstrates
499 that GSH alone does not allow characterization of the antiradical activity of YDs (Comuzzo et al.,
500 2015). But here, the observation of the activity of the other nucleophilic compounds allows better
501 understanding of the potential pool of compounds behind the antiradical activity. These results put
502 emphasis on the significance of a complex pool of nucleophilic compounds, rarely considered so
503 far, which contributes to the overall antioxidant activity of samples such as YDs.

504 **4. Conclusions**

505 The metabolomics approach provided evidence of specific fingerprints for YD soluble
506 fractions. The DPPH assay of these soluble fractions was performed in order to assess the YD
507 antiradical activity. The higher radical scavenging activity of yeast derivatives naturally rich in
508 GSH pointed out the positive influence of this specific production process on the antioxidant
509 activities of the YDs. Nevertheless, GSH concentrations appeared poorly correlated with the DPPH

510 scores implying the potential contribution of a larger pool of compounds from YDs to the oxidative
511 stability. The use of a model electrophile (4-methyl-1,2-benzoquinone) as a derivatization agent
512 revealed a pool of nucleophiles which may react with quinones in model wine; 52 nucleophiles
513 discriminated the YDs into four groups based on the number and the abundance of these reactive
514 compounds. This innovative separation of samples only based on derivatized compounds matched
515 very well with the DPPH scores, allowing samples to be ordered based on their stabilizing activity:
516 YD8 > YD7 > YD3 > YD1 > YD4 > YD6 > YD2. The UHPLC–Q-ToF-MS further enabled
517 annotation of most of the nucleophiles, which mostly belong to sulfur and nitrogen-sulfur
518 compounds. Our results confirmed the need to consider the whole chemical diversity of the
519 nucleophilic fraction present in the sample, beyond the sole GSH concentration. However,
520 monitoring the consumption rate of specific nucleophiles (for example GSH) can be used as an
521 indirect marker to estimate the activity of the entire pool of nucleophiles. Indeed, YDs with the
522 highest number of kinetically favorable nucleophilic reactions (longest GSH half-life in this
523 example) also appeared to possess the best antiradical activity. The important issue for the practical
524 application of GSH-enriched YDs in wine, would thus be that the slower the kinetic rate of the
525 GSH consumption (longer $t_{1/2}$), the higher the YD antioxidant potential, because wine could thus
526 benefit from a long-lasting reservoir of GSH antioxidant. This work opens new perspectives for
527 the analysis and development of yeast preparations dedicated to improving wine oxidative stability.

528 **Abbreviations**

529 4MeC, 4-methylcatechol; 4MeQ, 4-methyl-1,2-benzoquinone; DPPH, 2,2-diphenyl-1-
530 picrylhydrazyl; ESI, electrospray ionization; FTICR-MS, Fourier-transform ion cyclotron
531 resonance mass spectrometry; LOD, limit of detection; m/z , mass/charge; PCA, principal
532 component analysis; $R_{m20\%}$, mass ratio to reduce 20% initial absorbance; RT, retention time; SO₂,
533 sulfur dioxide (sulfite); $t_{1/2}$, half-life constant; UHPLC–Q-ToF-MS, ultra-high-performance liquid
534 chromatography coupled to quadrupole time-of-flight mass spectrometer; YD, yeast derivative

535

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542

543 **Conflicts of interest**

544 The authors wish to confirm that there are no conflicts of interest associated with this publication.

545

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687

688 **Figure Captions**

689

690 **Figure 1:** Mass of yeast derivatives needed to reduce the initial absorbance of DPPH by 20% (left
691 axis) and the corresponding concentration of glutathione in solution (right axis). Different letters
692 represent significant differences after pairwise Wilcoxon test: $n = 6$ and p -value < 0.01

693

694 **Figure 2:** Heatmap of the nucleophilic fingerprint of yeast derivatives (YDs). Clustering of YDs
695 based on the Euclidean distances between samples. Columns correspond to variables (named as
696 “ m/z _retention time (min)”) significantly increased after the addition of 4-methylquinone (Kruskal-
697 Wallis test, $n = 3$, p -value < 0.05). Grey color represents undetected compounds for a given YD.

698

699 **Figure 3:** PCA analysis (scores plot and correlation circle plot) of YD soluble fraction nucleophilic
700 fingerprints. The inner circle in the correlation circle plot corresponds to the correlation at 0.8.
701 Variables are named as “ m/z _retention time (min)”. The ellipse represents confidence level at 95%.
702 Variables in bold are glutathione ([428.1136_2.45], [508.0704_1.15], [733.1809_2.13],
703 [857.234_2.49]) or glutamyl-cysteine ([371.0922_2.55]) quinone addition products (m *Nu +
704 n *4MeC).

705

706 **Figure 4:** Representative kinetic profiles of nucleophiles consumption and nucleophile derivatives
707 production under chemical oxidation conditions (4MeC, Fe^{2+} , 30°C) during 40 h. Each point
708 represents the average of three replicates minus the average of the replicates at the initial time of
709 the oxidation, divided by the standard deviation (represented with the error bars). Lines correspond
710 to smoothed values of the area (calculated by Loess method).

711





