

Alma Mater Studiorum Università di Bologna  
Archivio istituzionale della ricerca

Quantification of 2- and 3-isopropylmalic acids in forty Italian wines by UHPLC-MS/MS triple quadrupole and evaluation of their antimicrobial, antioxidant activities and biocompatibility

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

*Published Version:*

Ricciutelli M., Bartolucci G., Campana R., Salucci S., Benedetti S., Caprioli G., et al. (2020). Quantification of 2- and 3-isopropylmalic acids in forty Italian wines by UHPLC-MS/MS triple quadrupole and evaluation of their antimicrobial, antioxidant activities and biocompatibility. FOOD CHEMISTRY, 321, 1-8 [10.1016/j.foodchem.2020.126726].

*Availability:*

This version is available at: <https://hdl.handle.net/11585/849864> since: 2022-01-31

*Published:*

DOI: <http://doi.org/10.1016/j.foodchem.2020.126726>

*Terms of use:*

Some rights reserved. The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>).  
When citing, please refer to the published version.

(Article begins on next page)

1 **Quantification of 2- and 3-isopropylmalic acids in forty Italian wines by**  
2 **UHPLC-MS/MS triple quadrupole and evaluation of their antimicrobial,**  
3 **antioxidant activities and biocompatibility**

4  
5 Massimo Ricciutelli<sup>1</sup>, Gianluca Bartolucci<sup>2</sup>, Raffaella Campana<sup>3</sup>, Sara Salucci<sup>3</sup>, Serena  
6 Benedetti<sup>3</sup>, Giovanni Caprioli,<sup>1\*</sup> Filippo Maggi<sup>1</sup>, Gianni Sagratini<sup>1</sup>, Sauro Vittori<sup>1</sup>, Simone  
7 Lucarini<sup>3\*</sup>

8  
9  
10 <sup>1</sup>School of Pharmacy, University of Camerino, Via Sant'Agostino 1, 62032 Camerino,  
11 Italy

12 <sup>2</sup>Department of Neurosciences Psychology, Drug Research and Child Health  
13 (NeuroFarBa), University of Florence, Florence, 50139, Italy

14 <sup>3</sup>Department of Biomolecular Sciences, Piazza del Rinascimento 6, University of Urbino  
15 Carlo Bo, 61029 Urbino, Italy

16  
17 **\*Corresponding authors:**

18 Giovanni Caprioli, School of Pharmacy, University of Camerino, via S.Agostino 1, 62032  
19 Camerino, Italy. Phone: +390737402238. Fax: +390737637345. Email:  
20 [giovanni.caprioli@unicam.it](mailto:giovanni.caprioli@unicam.it)

21 Simone Lucarini, Department of Biomolecular Sciences, Piazza del Rinascimento 6,  
22 University of Urbino Carlo Bo, 61029 Urbino, Italy. Phone: +390722303333. Fax:  
23 +390722303313. Email: [simone.lucarini@uniurb.it](mailto:simone.lucarini@uniurb.it)

24

25 **Abstract**

26 2-Isopropylmalic acid (2-IPMA) and 3-isopropylmalic acid (3-IPMA), recently discovered  
27 in wines, were simultaneously quantified in forty wines by UHPLC-MS/MS triple  
28 quadrupole. Principal component analysis displayed that red wines were more correlated  
29 with high amounts of 2-IPMA (average content 31.60 mg/L); white wines were mostly  
30 characterized by low levels of both organic acids. No correlation of their levels to other  
31 wine features (wine ageing or alcoholic content) were found. 2-IPMA and 3-IPMA showed  
32 MICs values of 4096 mg/L and MBCs values of 8192 mg/L or higher against several food  
33 borne pathogens. In association, an interesting lower MIC and MBC values (2048 mg/L  
34 and 4096 mg/L respectively) were observed against *Y. enterocolitica*. Interestingly, 3-  
35 IPMA showed a mild antioxidant activity by DPPH assay ( $EC_{50} = 3940$  mg/L), higher than  
36 that of 2-IPMA ( $EC_{50} > 4800$  mg/L). No toxicity of these compounds against human  
37 colorectal and liver cells (TB assay) was observed.

38

39

40 **Keywords:** wine, 2-isopropylmalic acid, 3-isopropylmalic acid, antimicrobial activity,  
41 antioxidant

42

43

44

## 45 **1. Introduction**

46 The category of wine flavour substances is composed of volatile compounds especially  
47 responsible for the odour and non-volatile components particularly contributing to the taste  
48 ([Whiting, 1976](#); [Lamikanra, Inyang & Leong, 1995](#)). Organic acids comprise one of the  
49 main taste groups, namely, that of sourness. The quantitatively dominating acids of grapes  
50 are maleic, tartaric, and citric acids, the first two of which account for over 90% of the total  
51 acid content of grapes ([Schreier & Jennings, 2013](#)). Wine is also rich in phenolic  
52 compounds, that are important components as they affect organoleptic characteristics, such  
53 as colour, astringency and aroma ([Kennedy, 2008](#)). Some years ago, [Ginjom et al. \(2011\)](#)  
54 identified and quantified the individual phenolic compounds present in wine at different  
55 wine-making stages but they were not able to identify two compounds, the first one  
56 (named U1) with a low molecular mass (176 u) and a large peak area eluting at around  
57 8.66 min, with a maximum absorbance at 275 nm; the second (named U2) with a high  
58 molecular mass (508 u) and a large peak area eluting at around 34.52 min, with a  
59 maximum absorbance at 360 nm. They noticed that the concentration (mg GAE/l) of U1  
60 increased during ageing, suggesting that it was continuously released either from the oak  
61 and/or from the degradation of other phenolics in the wine. The other unknown compound  
62 (U2) showed maximum concentration during oaking but decreased as the wine was bottle-  
63 aged. Both of these unknowns were independently reported by [Monagas et al. \(2005\)](#) and  
64 our group ([Salucci et al., 2017](#)).

65 In a recent paper, our research group has identified in wines not one but two isomeric  
66 compounds, corresponding to U1, with molecular weight of 176 u by using two LC-MS  
67 systems, i.e. LC-IT (ion trap) and LC-Q-Orbitrap ([Ricciutelli et al., 2019](#)). The two  
68 compounds were not chromatographically separated, as the quantification were performed  
69 by extracting the specific fragment of the two co-eluting isomers (i.e. m/z 115 or m/z 73)

70 and they were determined only in ten among red and white Italian wines. Thus, an  
71 improving of the chromatography separation would be desirable to better quantify these  
72 two molecules and more wine samples need to be analysed to correlate 2-IPMA and 3-  
73 IPMA levels with wine features. The molecular structure of the two molecules is reported  
74 in [Fig 1S](#). From literature, 2- and 3-IPMA are intermediates for the biosynthesis of leucine  
75 in yeast. In *Saccharomyces cerevisiae*, 2-IPMA is produced in mitochondria from  
76 isoketovalerate and then exported to the cytosol where it is isomerized to 3-IPMA. The  
77 latter is converted to leucine in two steps. Yeast cells naturally secrete these compounds into  
78 their surroundings ([Calvo, Kalyanpur, & Stevens 1962](#); [Dumlao, Hertz, & Clarke 2008](#) and  
79 [Marobbio, Giannuzzi, Paradies, Pierri, & Palmieri, 2008](#)). In particular, 2-IPMA secretion  
80 chelates aluminium ions and prevents them from entering to the cells, resulting in  
81 aluminium tolerance and reducing aluminum toxicity to the yeast cell ([Suzuki, Tamura,](#)  
82 [Nakanishi, Tashiro, Nishizawa, & Yoshimura, 2007](#)). The literature indicates that the  
83 concentrations of the active compounds in wines vary widely among the different grape  
84 cultivars from which they are derived. Indeed, it is well-recognized that biological  
85 activities and health benefits of wines depend on both the total amounts and the specific  
86 amounts of highly bioactive compounds ([Friedman, 2014](#)). To this regard, despite the  
87 evidence for the antimicrobial effectiveness of wine ([Moretro & Daeschel, 2004](#); [Carneiro,](#)  
88 [Couto, Mena, Queiroz, & Hogg, 2008](#); [Daglia, Papetti, Grisoli, Aceti, Dacarro, & Gazzani](#)  
89 [2007](#)), the contributions of specific wine components to the wine antimicrobial activity  
90 have not been well characterized ([Boban, Tonkic, Budimir, Modun, Sutlovic Punda-Polic](#)  
91 [&, Boban, 2010](#)). Different components of wine have been proposed to contribute to the  
92 antimicrobial activity, but the mechanisms responsible for this activity are not fully  
93 understood. In that regard, the reported investigations can be generally divided into two  
94 main groups, those giving emphasis to the role of wine phenolics and those accentuating

95 the role of nonphenolic constituents of wine (Friedman, 2014). The antioxidant activity and  
96 cytotoxicity of phenolic compounds in wine were exhaustively reported (Waterhouse,  
97 2002; Salucci et al., 2017 and Prasad, Jeyanthimala, & Ramachandran 2009). On the other  
98 hand, fewer reports could be found for acid components (Nelson, Rush, & Wilson, 2016;  
99 and Robles, Fabjanowicz, Chmiel, & Plotka-Wasyłka, 2019).

100 In the light of the scant information available in literature, the aims of the work were a) to  
101 develop a new and fast analytical method to simultaneously quantify 2- and 3-IPMA in  
102 forty wines (white and red ones) by using ultra-high-performance liquid chromatography–  
103 tandem mass spectrometry (UHPLC-MS/MS) triple quadrupole, b) to preliminary  
104 characterize the biological activity of these malic acid derivatives. In particular, the  
105 antibacterial and bactericidal activities of 2- and 3-IPMA, alone and in associations,  
106 against several foodborne pathogens were assessed. Moreover, antioxidant and  
107 biocompatibility studies were also carried out.

## 108 **2. Materials and Methods**

### 109 *2.1 Reagents and standards*

110 The analytical standards of 2-IPMA (CAS Number 3237-44-3) and 3-IPMA (CAS Number  
111 16048-89-8) were purchased from Sigma-Aldrich (Milano, Italy). The stock standard  
112 solution was prepared by dissolving 10 mg of each analyte in 10 mL of methanol and  
113 stored in a glass-stoppered bottle at 4°C in the dark. Standard working solutions, at various  
114 concentrations, were prepared daily by appropriate dilution of aliquots of the stock  
115 solutions in methanol. HPLC-grade acetonitrile and methanol were purchased from Sigma-  
116 Aldrich (Milan, Italy), while HPLC-grade formic acid 99-100% was bought from J.T.  
117 Baker B.V. (Deventer, Holland). For sample preparation and chromatographic analysis, de-  
118 ionized water of 18.2 MΩ/cm resistivity purified with a Milli-Q system (Millipore,

119 Bedford, USA) was used. All solvents were filtered through a 0.2  $\mu\text{m}$  polyamide filter  
120 from Sartorius Stedim (Goettingen, Germany) while all wine samples were filtered through  
121 a 0.2  $\mu\text{m}$  single use membrane syringe filter from Phenomenex (Bologna, Italy) before  
122 HPLC analysis.

### 123 *2.2 Wine samples*

124 Red and white wine samples were purchased in the supermarkets of the Camerino area  
125 (Camerino, Macerata, Italy).

### 126 *2.3 Sample preparation*

127 Briefly, 5 mL wine samples were extracted three times with ethyl acetate (5 mL) following  
128 and downscaling a previous published method (Ricciutelli et al., 2019; Salucci et al.,  
129 2017). The ethyl acetate extracts were pooled together prior to rotary evaporation at 30°C.  
130 The residue was re-dissolved in 0.25 mL methanol, filtrated through a 0.2  $\mu\text{m}$  membrane  
131 filter from Phenomenex (Bologna, Italy) and then directly injected into the HPLC-MS/MS.

### 132 *2.4 UHPLC-MS/MS analysis*

133 UHPLC–MS/MS studies were performed using an Agilent 1290 Infinity series and a Triple  
134 Quadrupole 6420 from Agilent Technology (Santa Clara, CA) equipped with an  
135 electrospray (ESI) source operating in negative ionization mode. The separation of  
136 polyphenols was achieved using a Zorbax RRHD C18 analytical column (50  $\times$  2.10 mm  
137 i.d., 1.8  $\mu\text{m}$ ), also from Agilent (USA). The mobile phase for UHPLC MS/MS analysis  
138 was a mixture of water (A, 97%) and acetonitrile (B, 3%), both with formic acid 0.1% at a  
139 flow rate of 0.4 mL  $\text{min}^{-1}$  with gradient elution. The solvent composition varied as  
140 follows: 0 min, 97% A; 5.5 min, 97% A; 5.5-14 min, 30% A; 14–19 min, 30% A; 19-24  
141 min, 97% A; until the end of the run at 30 min. The injection volume was 1  $\mu\text{L}$ . The  
142 temperature of the column was 30°C and the temperature of the drying gas in the  
143 ionization source was 300°C. The gas flow was 12 L  $\text{min}^{-1}$ , the nebulizer pressure was 50

144 psi and the capillary voltage was 4000 V. Detection was performed by electrospray  
145 ionization (ESI)-MS in the “multiple reaction monitoring” (Dynamic-MRM) mode. The  
146 selected ion transition and the settings of the mass analyzer are reported in **Table 1**. Before  
147 use, all solvents were filtered through a 0.2 µm filter from Sartorius Stedim (Goettingen,  
148 Germany), and before UHPLC analysis, all samples were filtered through a 0.2 µm single  
149 use syringe filter from Phenomenex (Bologna, Italy).

150 *2.5. Principal Component Analysis (PCA)* Forty samples of red and white wines were  
151 analyzed by PCA in order to identify possible correlation groups based on the content of  
152 the two isomers of isopropyl-malic acid. A covariance matrix based on 40 samples x 2  
153 variables was created and data were analyzed by STATISTICA v.7.1 (Stat Soft Italia S.r.l.,  
154 Vigonza, Italy). Score and loading plots were generated by calculating eigenvalues.

#### 155 *2.6 Bacterial strains and culture conditions*

156 Five reference human pathogens were used in this study: *Escherichia coli* O157:H7 ATCC  
157 35150, *Listeria monocytogenes* ATCC 7644, *Staphylococcus aureus* ATCC 29213,  
158 *Salmonella enterica* ATCC 13314 and *Yersinia enterocolitica* ATCC 14053. The strains  
159 were routinely grown on Tryptic Soy Agar (TSA, VWR, Milan, Italy) at 37 °C for 24 h.  
160 All the stock cultures were kept at -80 °C in Nutrient broth (Oxoid, Italy) with 15% of  
161 glycerol.

#### 162 *2.7. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal 163 concentration (MBC)*

164 MICs of all the tests compounds were determined by standard micro-dilution method.  
165 First, for each compound a stock solution was prepared in one mL of distilled water and  
166 subsequently sterilized by 0.22 µm pore size filters (VWR). Several colonies of each  
167 bacterial strain were inoculated in 10 mL of sterile Mueller-Hinton broth (MHB) (VWR)  
168 and incubated at 37 °C for 18 h. At the end of incubation, each bacterial suspension was



169 adjusted by spectrophotometer to about  $10^6$  cfu/mL (OD 610nm 0.13-0.15) and 100  $\mu$ L  
170 was added in wells of the 96-well plate together with the appropriate volumes of the test  
171 solutions (final concentration from 256 to 8192 mg/L). In addition, 2-IPMA and 3-IPMA  
172 were also tested in association (ratio 10:1 and 1:10). For the association, the analyzed  
173 concentrations are the sum of the two acids contribution in the correct ratio. For example,  
174 total concentration = 4096  $\mu$ g/mL of 2-IPMA:3-IPMA (10:1 ratio) is the sum of 3724  $\mu$ g of  
175 2-IPMA (10 parts) and 372  $\mu$ g of 3-IPMA (1 part) in 1 mL of solution. Two rows were  
176 used for positive (bacteria alone) and negative controls (MHB alone), respectively.  
177 Amoxicillin and gentamicin (1-128 mg/L) (Sigma, Italy), were used and internal control.  
178 After 24h of incubation at 37°C, the plates were observed and the MIC was determined as  
179 the lowest concentration that inhibits the visible growth in comparison with the control  
180 samples. The optical density (600 nm) of each well was also assessed using a Multiscan Ex  
181 Microplate Reader (Thermo Scientific, Italy). All data were expressed as the mean of three  
182 independent experiments performed in duplicate. For MBC determination, ten microliters  
183 from the invisible growth wells were inoculated in triplicate on TSA and incubated at 37  
184 °C for 24 h; MBC was defined as the lowest concentration of each compound that  
185 completely inhibited growth on TSA (approximately 99.5% of killed microorganisms).  
186 All the experiments were performed in duplicate.

#### 187 *2.8. DPPH radical scavenging activity*

188 The antioxidant activity of 2-IPMA, 3-IPMA and Malic acid (MA) was evaluated using the  
189 DPPH assay, which measures the ability of a compound to act as free radical scavenger or  
190 hydrogen donor (Sagar B. Kedare and R. P. Singh, 2011). Briefly, 0.300 mL of sample  
191 diluted in ethanol (range 0.3-9.6 mg/mL) was added to 0.300 mL of 100  $\mu$ M DPPH in  
192 ethanol. Absorbance decrease at 517 nm was recorded after 30 min at room temperature.  
193 The scavenger effect was calculated as  $\% = [(Abs\ 517\ nm\ of\ blank - Abs\ 517\ nm\ of$

194 sample /Abs 517 nm of blank] x 100. EC<sub>50</sub> (i.e. the concentration required to obtain a 50%  
195 antioxidant effect) was also calculated. Ascorbic acid (initial concentration 0.3-9.6 µg/mL)  
196 was used as standard to check the correctness of the procedures. Statistical significance  
197 was assessed by one-way ANOVA and Tukey's posthoc test for multiple comparisons  
198 (PRISM 6, GraphPad Software, USA). Significance level was set at p < 0.05.

### 199 *2.9. Human cell culture and treatments (Trypan Blue exclusion assay)*

200 The human colorectal Caco-2 and the liver HepG2 cancer cells, were seeded on multiwell  
201 plates at a cell density of  $1 \times 10^6$  cells/mL and were cultured in a DMEM high glucose  
202 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM  
203 glutamine, 1% penicillin-streptomycin, and MEM nonessential amino acid solution (1X).

204 All culture models were maintained at 37°C in humidified air with 5% CO<sub>2</sub> and cell  
205 behavior were monitored by means of an Inverted Microscopy (Eclipse TE2000-S Nikon;  
206 objective 10x). Cells were treated with different doses of 2-IPMA, 3-IPMA and MA to  
207 verify their potential toxic effect. 2-IPMA, 3-IPMA and MA were added to the culture  
208 medium (and monitored until 48h) at the following final concentrations: 1.76, 8.8, 35.2, 88,  
209 176, 880 and 1176 mg/L. Moreover, 2-IPMA e 3-IPMA have been administrated to cell  
210 cultures in association (ratio 10:1 and 1:10) using these dosages: 1.76 mg/L + 15.84 mg/L,  
211 35.2 mg/L + 316.8 mg/L, 176 mg/L + 1584 mg/L. The treatments were compared with 50  
212 µM etoposide, a known chemoterapic drug (Salucci et al., 2014). Trypan Blue (TB)  
213 exclusion assay was employed to monitor cell viability after all treatments since it  
214 represents a useful method to quantify live and dead cell number (Salucci et al., 2018).

215 In addition, cells have been exposed to 0.5 mM hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) for 24 h, a pro-  
216 oxidant agent and cell death trigger (Salucci et al., 2014). MA, 2-IPMA, 3-IPMA alone and  
217 the association of 2- and 3-IPMA (ratio 10:1 and 1:10) have been administrated to cells for  
218 24 h before H<sub>2</sub>O<sub>2</sub> treatment to verify their potential protection against oxidative damage.

219 Also in this case, TB exclusion assay has been used to monitor cell viability for both  
220 human colorectal Caco-2 and the liver HepG2 cancer cell lines.

221 Data collected from each experimental condition were presented as mean of living cell  
222 percentage  $\pm$  standard deviation of the mean (SEM).

### 223 **3 Results and discussion**

#### 224 *3.1 Method validation of the HPLC-MS/MS method*

225 The investigated performance characteristics of the developed method were linearity, limit  
226 of detection (LOD), limit of quantification (LOQs) (**Table 2**), and matrix effect (as signal  
227 suppression/enhancement %) (**Table 1S**). Linearity was tested by injecting 7 different  
228 concentrations of authentic standard mixtures of the analytes in solvent (**Table 2**) from 0.5  
229 to 100 mg/L. Calibration curves (concentrations versus peak areas) were determined by  
230 least-squares regression analysis obtaining correlation coefficients ( $R^2$ ) higher than 0.9942.  
231 The reproducibility of the chromatographic retention times was examined five times over a  
232 five-day period ( $n=25$ ) obtaining high stability (RSD < 1.3%). The LODs and LOQs were  
233 estimated on the basis of 3:1 and 10:1 signal-to-noise ratios obtained with standards  
234 containing the compounds at low concentration levels. The estimated LODs and LOQs  
235 were 0.01 and 0.02 mg/L and 0.03 and 0.06 mg/L for 2-IPMA and 3-IPMA respectively  
236 (**Table 2**).

237 Accuracy was calculated via spike-recovery and it was determined using percentage  
238 relative errors at two different concentrations by comparing the true value of the analyte in  
239 the sample (true concentration) with the values obtained by analysis (measured  
240 concentration). As it can be seen from **Table 2S** in supplementary materials, the % relative  
241 error for the standards are between + 2.9 and +10.4 % for the 2 different analytes. The  
242 lowest % relative error is (+2.9%) obtained for 2-IPMA at concentration level of 50 mg/L,

243 meanwhile the highest % relative error is +10.4% obtained for 3-IPMA at concentration  
244 level of 5 mg/L. The measure of precision usually is expressed in terms of imprecision and  
245 computed as standard deviation of the test result. The precision of this method was  
246 determined by monitoring the RSD % of the three replicates. As it can be seen from **Table**  
247 **2S** reported in supplementary materials, all the RSD % obtained are between 2.8 – 5.9% .  
248 The lowest RSD was 2.8% for 2-IPMA at concentration level of 50 mg/L, while the  
249 highest RSD was 4.9% for 3-IPMA at concentration level of 5 mg/L.

250 Matrix effect was studied comparing the slope of a calibration curve in pure solvent and of  
251 a matrix-matched curve prepared by adding the standards to a wine extract (after  
252 extraction). The calibration curve was obtained by subtracting in each concentration the  
253 amount of polyphenols obtained from the blank extract. These curves were obtained after  
254 injecting seven concentration levels, between 5 and 250 mg/L. The slope of the standard  
255 addition plot was compared with the slope of standard calibration plot to evaluate the  
256 matrix effects. The signal suppression/enhancement (SSE) was calculated according to the  
257 following equation (Caprioli, Nzekoue, Giusti, Vittori & Sagratini, 2018):

$$258 \text{ SSE \%} = (\text{slope matrix matched curve/slope pure solvent curve}) \times 100;$$

259 If SSE (%) is about 100% there is no matrix effect, values < 100% indicate signal  
260 suppression, while values > 100% indicate signal enhancement. In our study the SSE %  
261 ranged from 46 (2-IPMA) to 59 (3-IPMA) indicating a negative matrix effect (ion  
262 suppression) of 54 and 41%, respectively (**Table 1S**). In the following paragraph, the  
263 quantification of the two analytes in wine samples have been reported taken into account  
264 ME studies.

### 265 *3.2 Quantification of 2-IPMA and 3-IPMA in Italian wines*

266 The validated UHPLC-MS/MS triple quadrupole method was used to analyse 40 Italian  
267 wine samples, specifically 19 red and 21 white wines and the two analytes were found  
268 in all samples (**Table 3**). **Fig. 2S** show an HPLC-MS/MS chromatogram of a red wine  
269 samples with the TIC (total ion chromatogram) and the multiple reaction monitoring  
270 (MRM) transition of the two organic acids. This method offers significant improvement  
271 with respect to our previous work ([Ricciutelli et al., 2019](#)). The Zorbax RRHD C18  
272 analytical column (50 × 2.10 mm i.d., 1.8 μm) provided good performance in terms of  
273 resolution and selectivity as the two isomers were perfectly separated (please see **Fig.**  
274 **2S**). Moreover, LODs and LOQs obtained are at least 10 times lower than values  
275 reported in our previous article ([Ricciutelli et al., 2019](#)). The concentration of the two  
276 analytes has been expressed in mg/L in the original wine samples. Concerning red  
277 wines, the contents of 2-IPMA ranged from 18.9 mg/L of Montepulciano (sample n°1)  
278 to 41.6 mg/L of Merlot (n°19). Nero Buono (n°18), Montepulciano D’Abruzzo (n°17)  
279 and Vernaccia di Serrapetrona (n°16) wines displayed a conspicuous content of 2-IPMA  
280 with levels of 41.25, 38.25 and 37.63 mg/L, respectively. On the other hand, 3-IPMA  
281 was found in low concentration ranging from 1.43 mg/L in Montepulciano (n°1) to 4.13  
282 mg/L of Merlot (n°19). The average content of 2-IPMA in the 19 wines was 31.60  
283 mg/L, whereas that of 3-IPMA was 1.65 mg/L.

284 Concerning the white wines, the contents of the two derivatives of malic acid are a bit  
285 lower respect to red ones. In fact, 2-IPMA ranged from 6.7 mg/L of Primofiore (n°20)  
286 to 27.8 mg/L of Verdicchio di Matelica (sample n°40). Noteworthy levels of 2-IPMA  
287 were found also in Bellone Anfora sample n°39 (26.00 mg/L) and Verdicchio di  
288 Matelica sample n°38 (25.82 mg/L). As reported for red wines, the amounts of 3-IPMA  
289 detected in white wines were lower with respect to 2-IPMA. The content of 3-IPMA

290 ranged from 0.56 mg/L of Primofiore (n°20) to 2.42 mg/L of Verdicchio di Matelica  
291 n°40 (2.42 mg/L). Bellone (n°34), Verdicchio di Matelica (n°38) and Muller Thurgaut  
292 (n°37) wines displayed a conspicuous content of 3-IPMA with levels of 1.91, 1.63 and  
293 1.57 mg/L, respectively. The average contents of 2-IPMA and 3-IPMA in white wines  
294 were 19.57 mg/L and 0.93 mg/L, respectively.

295 PCA analysis allowed to visualize two main groups of wine samples as depicted in **Fig.**  
296 **1**. 2-IPMA was the main variable influencing data variability in the first principal  
297 component (99.6%, values of eigenvectors: 8.42; -0.03). They were represented mostly  
298 by red wine samples on the right-hand side of the score plot that were more correlated  
299 with high amounts of 2-IPMA. On the other hand, white wine samples were mostly in  
300 the left-hand side of the score plot and were characterized by low levels of both  
301 organic acids.

### 302 *3.3 Antimicrobial activity of 2-IPMA and 3-IPMA*

303 The assessment of the antibacterial activity of 2-IPMA and 3-IPMA against five human  
304 pathogens was performed according to the National Committee for Clinical Laboratory  
305 Standards (NCCLS) document M100-S12 method (**Table 4**). As shown, MICs values of  
306 4096 mg/L were determined for both the compounds for both gram-positive (*L.*  
307 *monocytogenes* ATCC 7644 and *S. aureus* ATCC 29213) and gram-negative (*E. coli*  
308 O157:H7 ATCC 35150, *S. enterica* ATCC 13314 and *Y. enterocolitica* ATCC 14053)  
309 bacteria. As regards the bactericidal activity, most of the bacterial strains were killed by 2-  
310 IPMA and 3-IPMA at the concentration of 8192 mg/L, though in some cases higher MBCs  
311 values (>8192 mg/L) were observed. The association 2-IPMA:3-IPMA in the ratio 10:1 did  
312 not show remarkable differences in MICs values, resulting of 4096 mg/L for all the  
313 microorganisms. Similarly, no differences in MICs values were evidenced using 2-

314 IPMA:3-IPMA in the ratio 1:10 (MIC 4096 mg/L), with the only exception of *Y.*  
315 *enterocolitica* ATCC 14053 for which a lower MIC (2048 mg/L) was noted with an  
316 interesting synergistic effect. As regards the bactericidal activity of the mentioned  
317 combinations, MBCs >4096 mg/L were observed for *E. coli* O157:H7 ATCC 35150, *L.*  
318 *monocytogenes* ATCC 7644, *S. aureus* ATCC 29213 and *S. enterica* ATCC 13314, while  
319 the MBC value for *Y. enterocolitica* ATCC 14053 resulted to be 4096 mg/L. The MICs of  
320 the internal controls showed the susceptibility of all the examined bacteria to Gentamicin,  
321 with MICs ranging from 4 to 16 mg/L, as previously observed (Campana et al., 2019;  
322 Perinelli et al., 2018, Mengist et al., 2018), while a lower susceptibility was detected for  
323 Amoxicillin (from 128 to >128 mg/L) (Obaidat and Stringer 2019; Mengist et al., 2018).

#### 324 3.4. Antioxidant activity of 2-IPMA and 3-IPMA by DPPH radical scavenging assay

325 Among the test compounds, 3-IPMA showed the highest scavenging activity towards  
326 DPPH ( $EC_{50} = 3.94 \pm 0.13$  mg/mL), followed by 2-IPMA and MA ( $EC_{50} > 4.8$  mg/mL for  
327 both compounds) (Figure 2A). Ascorbic acid, used as reference compound, presented an  
328  $EC_{50}$  value of  $1.64 \pm 0.11$   $\mu$ g/mL (Data not shown). 2-IPMA and MA show almost  
329 comparable no anti-oxidant activities, however 3-IPMA shows a very mild anti-oxidant  
330 activity. This property could be attributed to the relative stability of the tertiary radical  
331 formed with DPPH compared to the secondary radical formed from 2-IPMA (see Figure  
332 1S, supplementary file).

#### 333 3.5. Toxicity studies of 2-IPMA and 3-IPMA by TB exclusion assay

334 2-IPMA, 3-IPMA and MA administered to cell models and monitored until 48h, were not  
335 cytotoxic for colon and liver human cells and did not affect their viability. In fact, TB  
336 assay revealed that the number of living cells after all treatments is comparable to that

337 quantified in control condition. The same results can be observed by exposing cells to 2-  
338 IPMA and 3-IPMA in association. On the contrary, after etoposide exposure an evident  
339 decrease of living cells were observed. These findings clearly appear in **Figure 2B** and  
340 **Figure 3S** (see supplementary file) that show no toxicity of the tested acids in all the  
341 concentration for HepG2 and Caco-2 cells viability after 24 h of treatments, respectively.  
342 The absence of cytotoxicity is confirmed when the 2- and 3-IPMA are used in association  
343 (10:1 and 1:10 ratio, **Figure 4S** in supplementary file). Furthermore, 2- and 3-IPMA used  
344 singly or in combination do not evidence any protection against cell death induced by the  
345 oxidative damage of hydrogen peroxyde (data not shown).

346

#### 347 **4. Conclusions**

348 A new, sensitive and fast analytical method to simultaneously quantify 2-IPMA and 3-  
349 IPMA in forty wines was developed and validated by using UHPLC-MS/MS triple  
350 quadrupole. The two analytes were found in all wine samples analyzed; the average  
351 content of 2-IPMA was 31.60 mg/L in red wines and 19.57 mg/L in white wines; whereas  
352 that of 3-IPMA was 1.65 mg/L and 0.93 mg/L, respectively. Statistical analyses displayed  
353 that red wine samples seem to be correlated with high amounts of 2-IPMA; on the other  
354 hand, white wine samples were mostly characterized by low levels of both organic acids.  
355 The two isomeric compounds were tested alone and in association against five  
356 representative food borne pathogens for MIC and MBC determination. 2-IPMA and 3-  
357 IPMA showed mild antibacterial and bactericidal activity, having MICs values of 4096  
358 mg/L (23.3 mM) and MBCs values of 8192 mg/L (46.5 mM) or higher for both gram-  
359 positive (*L. monocytogenes* and *S. aureus*) and gram-negative (*E. coli*, *S. enterica* and *Y.*  
360 *enterocolitica*) bacteria. In association, 2-IPMA and 3-IPMA did not show remarkable



361 differences in MICs and MBCs values. However, using 2-IPMA together with 3-IPMA in  
362 the ratio 1:10, an interesting lower MIC and MBC (2048 mg/L and 4096 mg/L  
363 respectively) were observed against *Y. enterocolitica*, highlighting a possible synergistic  
364 effect of the two isomeric acids against this gram-negative strain. Interestingly, 3-IPMA  
365 showed a mild antioxidant activity by DPPH assay ( $EC_{50} = 3940$  mg/L, 22.4 mM) higher  
366 than that of its isomer 2-IPMA ( $EC_{50} > 4800$  mg/L). No toxicity of these compounds alone  
367 and in association was observed at all tested concentrations as evidenced by TB exclusion  
368 assay against human colorectal and liver cell lines. The mild antibacterial and mild  
369 antioxidant activities together with the good biocompatibility made 2- and 3-IPMA  
370 interesting candidates as preservatives for foods.

#### 371 **Conflict of Interests**

372 The authors declare that no competing interests exist.

373

#### 374 **References**

375 Boban, N., Tonkic, M., Budimir, D., Modun, D., Sutlovic, D., Punda-Polic, V., & Boban  
376 M. (2010). Antimicrobial effects of wine: separating the role of polyphenols, pH, ethanol,  
377 and other wine components. *Journal of Food Science*, 75(5), M322-6.

378 Calvo, M. J., Kalyampur, M. G., & Stevens C. M. (1962). 2-Isopropylmalate and 3-  
379 isopropylmalate as intermediates in leucine biosynthesis. *Biochemistry*, 6, 1157-1161.

380 Campana, R., Merli, A., Verboni, M., Biondo, F., Favi, G., Duranti, A., & Lucarini S.  
381 (2019). Synthesis and evaluation of saccharide-based aliphatic and aromatic esters as  
382 antimicrobial and antibiofilm agents. *Pharmaceuticals*, 12 (4), 186.

383 Caprioli, C., Nzekoue, F., Giusti, F., Vittori, S., & Sagratini G. (2018). Optimization of an  
384 extraction method for the simultaneous quantification of sixteen polyphenols in thirty-one  
385 pulse samples by using HPLC-MS/MS dynamic-MRM triple quadrupole. *Food Chemistry*,  
386 266, 490-497.

387 Carneiro, A, Couto, J.A., Mena, C., Queiroz, J., & Hogg, T. (2008). Activity of wine  
388 against *Campylobacter jejuni*. *Food Control*, 19, 800–805.

389 Daglia, M., Papetti, A., Grisoli, P., Aceti, C., Dacarro, C., & Gazzani G. (2007).  
390 Antibacterial activity of red and white wine against oral streptococci. *Journal of*  
391 *Agricultural and Food Chemistry*, 55, 5038–42.

392 Dumlao, S. D., Papetti, A., Hertz, N., & Clarke G. (2008). Secreted 3-isopropylmalate  
393 methyl ester signals invasive growth during amino acid starvation in *Saccharomyces*  
394 *cerevisiae*. *Biochemistry*, 47, 698–709.

395 Friedman, M. (2014). Antibacterial, antiviral, and antifungal properties of wines and  
396 winery byproducts in relation to their flavonoid content. *Journal of Agricultural and Food*  
397 *Chemistry*, 62, 6025–6042.

398 Ginjom, I., D’Arcy, B., Caffin, N., & Gidley, M. (2011). Phenolic compound profiles in  
399 selected Queensland red wines at all stages of the wine-making process. *Food Chemistry*  
400 125, 823–834.

401 Lamikanra, O., Inyang I. D., & Leong, S. (1995). Distribution and effect of grape maturity  
402 on organic acid content of red muscadine grapes. *Journal of Agricultural and Food*  
403 *Chemistry*, 43, 3026-3028

404 Kennedy, J. A. (2008). Grape and wine phenolics: Observations and recent findings.  
405 *Ciencia E Investigacion Agraria*, 35, 107–120.

406 Marobbio, C. M. T., Giannuzzi, G., Paradies, E., Pierri, C. L., & Palmieri, F. (2008).  $\alpha$ -  
407 Isopropylmalate, a leucine biosynthesis intermediate in yeast, is transported by the  
408 mitochondrial oxalacetate carrier. *Journal of Biological Chemistry*, 17, 283, 28445–28453.

409 Mengist, A., Mengistu, G., & Reta, A. (2018). Prevalence and antimicrobial susceptibility  
410 pattern of salmonella and shigella among food handlers in catering establishments at Debre  
411 Markos University, Northwest Ethiopia. *International Journal of Infectious Diseases*, 75,  
412 74-79.

413 Monagas, M., Suarez, R., Gomez-Cordoves, C., & Bartolome, B. (2005). Simultaneous  
414 determination of nonanthocyanin phenolic compounds in red wines by HPLC/DAD/ESI-  
415 MS. *American Journal of Enology and Viticulture*, 56, 139–147.

416 Moretro, T., & Daeschel, MA. (2004). Wine is bactericidal to foodborne pathogens.  
417 *Journal of Food Science*, 69, 251–7.

418 Nelson, H. N., Rush, K. L., & Wilson, T. (2016). Functions of common beverage  
419 ingredients. In Wilson T., Temple N. (Eds.), *Beverage Impacts on Health and Nutrition*.  
420 *Nutrition and Health*. (pp. 317–329). New York: Humana Press, Cham.

421 Obaidat, M.M., & Stringer, A.P. (2019). Prevalence, molecular characterization, and  
422 antimicrobial resistance profiles of *Listeria monocytogenes*, *Salmonella enterica*, and  
423 *Escherichia coli* O157:H7 on dairy cattle farms in Jordan. *Journal of Dairy Science*, 102,  
424 8710-8720.

425 Perinelli, D.R., Lucarini, S., Fagioli, L., Campana, R., Villasaliu, D., Duranti, A., &  
426 Casettari, L. (2018). Lactose oleate as new biocompatible surfactant for pharmaceutical  
427 applications. *European Journal of Pharmaceutics and Biopharmaceutics*, 124, 55-62.

428 Prasad, N. R, Jeyanthimala, K, & Ramachandran, S. (2009). Caffeic acid modulates  
429 ultraviolet radiation-B induced oxidative damage in human blood lymphocytes. *J.*  
430 *Photochem. Photobiol. B*, 95, 196–203.

431 Ricciutelli, M., Moretti, S., Galarini, R., Sagratini, G., Mari, M., Lucarini, S., Vittori, S.,  
432 Caprioli, G. (2019). Identification and quantification of new isomers of isopropyl-malic  
433 acid in wine by LC-IT and LC-Q-Orbitrap. *Food Chemistry*, 294, 390-396.

434 Robles, A., Fabjanowicz, M., Chmiel, T., & Plotka-Wasyłka, J. (2019). Determination and  
435 identification of organic acids in wine samples. Problems and challenges. *Trends in*  
436 *Analytical Chemistry*, 120, 115630.

437 Sagar B. Kedare and R. P. Singh (2011). Genesis and development of DPPH method of  
438 antioxidant assay. *J Food Sci Technol.*, 48, 412–422.

439 Salucci S., Burattini S, Battistelli M., Baldassarri V., Curzi D., Valmori A., & Falcieri E.  
440 (2014). Melatonin prevents chemical-induced haemopoietic cell death. *International*  
441 *Journal of Molecular Sciences*, 15 (4), 6625-6640.

442 Salucci S., Burattini S., Giordano F. M., Lucarini S., Diamantini G., & Falcieri E. (2017).  
443 Further highlighting on the prevention of oxidative damage by polyphenol-rich wine  
444 extracts. *Journal of Medicinal Food*, 20, 410–419.

445 Salucci S., Burattini S, Buontempo F., Orsini E., Furiassi L., Mari M., Lucarini S., Martelli  
446 A. M., & Falcieri E. (2018). Marine bisindole alkaloid: A potential apoptotic inducer in  
447 human cancer cells. *European Journal of Histochemistry*, 62 (2), 2881.

448 Schreier, P., & Jennings, W. G. (1979). Flavor composition of wines: A review, C R C  
449 *Critical Reviews in Food Science and Nutrition*, 12, 59-111.

450 Suzuki, T., Tamura, S., Nakanishi, H., Tashiro, M., Nishizawa, N. K., & Yoshimura, E.  
451 (2007). Reduction of aluminum toxicity by 2-IPMA acid in the budding yeast  
452 *Saccharomyces cerevisiae*. *Biological Trace Element Research*, 120, 257-263.

453 Waterhouse, A. L. (2002). Wine phenolics. *Ann NY Acad Sci*, 957, 21-36.

454 Whiting, G. C. (1976). Organic acid metabolism of yeasts during fermentation of alcoholic  
455 beverages. A review. *Journal of the Institute of Brewing*, 82, 84-92.