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

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Abstract

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

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

1. Introduction

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

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

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

Bedford, USA) was used. All solvents were filtered through a 0.2 µm polyamide filter from Sartorius Stedim (Goettingen, Germany) while all wine samples were filtered through a 0.2 µm single use membrane syringe filter from Phenomenex (Bologna, Italy) before HPLC analysis.

2.2 Wine samples

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

2.3 Sample preparation

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

2.4 UHPLC-MS/MS analysis

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

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

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

2.6 Bacterial strains and culture conditions

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

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

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

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

2.8. DPPH radical scavenging activity

The antioxidant activity of 2-IPMA, 3-IPMA and Malic acid (MA) was evaluated using the DPPH assay, which measures the ability of a compound to act as free radical scavenger or hydrogen donor (Sagar B. Kedare and R. P. Singh, 2011). Briefly, 0.300 mL of sample diluted in ethanol (range 0.3-9.6 mg/mL) was added to 0.300 mL of 100 µM DPPH in ethanol. Absorbance decrease at 517 nm was recorded after 30 min at room temperature.

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

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

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

All culture models were maintained at 37°C in humidified air with 5% CO_2 and cell behavior were monitored by means of an Inverted Microscopy (Eclipse TE2000-S Nikon; objective 10x). Cells were treated with different doses of 2-IPMA, 3-IPMA and MA to verify their potential toxic effect. 2-IPMA, 3-IPMA and MA were added to the culture medium (and monitored until 48h) at the following final concentrations: 1.76, 8.8, 35.2, 88, 176, 880 and 1176 mg/L. Moreover, 2-IPMA and 3-IPMA have been administrated to cell cultures in association (ratio 10:1 and 1:10) using these dosages: 1.76 mg/L + 15.84 mg/L, 35.2 mg/L + 316.8 mg/L, 176 mg/L + 1584 mg/L. The treatments were compared with 50 µM etoposide, a known chemoterapic drug (Salucci et al., 2014). Trypan Blue (TB) exclusion assay was employed to monitor cell viability after all treatments since it represents a useful method to quantify live and dead cell number (Salucci et al., 2018).

In addition, cells have been exposed to 0.5 mM hydrogen peroxide (H_2O_2) for 24 h, a pro-oxidant agent and cell death trigger (Salucci et al., 2014). MA, 2-IPMA, 3-IPMA alone and the association of 2- and 3-IPMA (ratio 10:1 and 1:10) have been administrated to cells for

24 h before H_2O_2 treatment to verify their potential protection against oxidative damage. Also in this case, TB exclusion assay has been used to monitor cell viability for both human colorectal Caco-2 and the liver HepG2 cancer cell lines.

Data collected from each experimental condition were presented as mean of living cell percentage ± standard deviation of the mean (SEM).

3 Results and discussion

3.1 Method validation of the HPLC-MS/MS method

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

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

lowest % relative error is (+2.9%) obtained for 2-IPMA at concentration level of 50 mg/L, meanwhile the highest % relative error is +10.4% obtained for 3-IPMA at concentration level of 5 mg/L. The measure of precision usually is expressed in terms of imprecision and computed as standard deviation of the test result. The precision of this method was determined by monitoring the RSD % of the three replicates. As it can be seen from **Table 2S** reported in supplementary materials, all the RSD % obtained are between 2.8 – 5.9% . The lowest RSD was 2.8% for 2-IPMA at concentration level of 50 mg/L, while the highest RSD was 4.9% for 3-IPMA at concentration level of 5 mg/L.

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

SSE % = (slope matrix matched curve/slope pure solvent curve)×100;

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

266 *3.2 Quantification of 2-IPMA and 3-IPMA in Italian wines*

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

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

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

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

303 *3.3 Antimicrobial activity of 2-IPMA and 3-IPMA*

304 The assessment of the antibacterial activity of 2-IPMA and 3-IPMA against five human
305 pathogens was performed according to the National Committee for Clinical Laboratory
306 Standards (NCCLS) document M100-S12 method (**Table 4**). As shown, MICs values of
307 4096 mg/L were determined for both the compounds for both gram-positive (*L.*
308 *monocytogenes* ATCC 7644 and *S. aureus* ATCC 29213) and gram-negative (*E. coli*
309 O157:H7 ATCC 35150, *S. enterica* ATCC 13314 and *Y. enterocolitica* ATCC 14053)
310 bacteria. As regards the bactericidal activity, most of the bacterial strains were killed by 2-
311 IPMA and 3-IPMA at the concentration of 8192 mg/L, though in some cases higher MBCs
312 values (>8192 mg/L) were observed. The association 2-IPMA:3-IPMA in the ratio 10:1 did
313 not show remarkable differences in MICs values, resulting of 4096 mg/L for all the

314 microorganisms. Similarly, no differences in MICs values were evidenced using 2-
315 IPMA:3-IPMA in the ratio 1:10 (MIC 4096 mg/L), with the only exception of *Y.*
316 *enterocolitica* ATCC 14053 for which a lower MIC (2048 mg/L) was noted with an
317 interesting synergistic effect. As regards the bactericidal activity of the mentioned
318 combinations, MBCs >4096 mg/L were observed for *E. coli* O157:H7 ATCC 35150, *L.*
319 *monocytogenes* ATCC 7644, *S. aureus* ATCC 29213 and *S. enterica* ATCC 13314, while
320 the MBC value for *Y. enterocolitica* ATCC 14053 resulted to be 4096 mg/L. The MICs of
321 the internal controls showed the susceptibility of all the examined bacteria to Gentamicin,
322 with MICs ranging from 4 to 16 mg/L, as previously observed (Campana et al., 2019;
323 Perinelli et al., 2018, Mengist et al., 2018), while a lower susceptibility was detected for
324 Amoxicillin (from 128 to >128 mg/L) (Obaidat and Stringer 2019; Mengist et al., 2018).

325 *3.4. Antioxidant activity of 2-IPMA and 3-IPMA by DPPH radical scavenging assay*

326 Among the test compounds, 3-IPMA showed the highest scavenging activity towards
327 DPPH (EC_{50} = 3.94 ± 0.13 mg/mL), followed by 2-IPMA and MA (EC_{50} > 4.8 mg/mL for
328 both compounds) (**Figure 2A**). Ascorbic acid, used as reference compound, presented an
329 EC_{50} value of 1.64 ± 0.11 µg/mL (Data not shown). 2-IPMA and MA show almost
330 comparable no anti-oxidant activities, however 3-IPMA shows a very mild anti-oxidant
331 activity. This property could be attributed to the relative stability of the tertiary radical
332 formed with DPPH compared to the secondary radical formed from 2-IPMA (see **Figure**
333 **1S**, supplementary file).

334 *3.5. Toxicity studies of 2-IPMA and 3-IPMA by TB exclusion assay*

335 2-IPMA, 3-IPMA and MA administered to cell models and monitored until 48h, were not
336 cytotoxic for colon and liver human cells and did not affect their viability. In fact, TB

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

4. Conclusions

A new, sensitive and fast analytical method to simultaneously quantify 2-IPMA and 3-IPMA in forty wines was developed and validated by using UHPLC-MS/MS triple quadrupole. The two analytes were found in all wine samples analyzed; the average content of 2-IPMA was 31.60 mg/L in red wines and 19.57 mg/L in white wines; whereas that of 3-IPMA was 1.65 mg/L and 0.93 mg/L, respectively. Statistical analyses displayed that red wine samples seem to be correlated with high amounts of 2-IPMA; on the other hand, white wine samples were mostly characterized by low levels of both organic acids. The two isomeric compounds were tested alone and in association against five representative food borne pathogens for MIC and MBC determination. 2-IPMA and 3-IPMA showed mild antibacterial and bactericidal activity, having MICs values of 4096 mg/L (23.3 mM) and MBCs values of 8192 mg/L (46.5 mM) or higher for both gram-positive (*L. monocytogenes* and *S. aureus*) and gram-negative (*E. coli*, *S. enterica* and *Y.*

enterocolitica) bacteria. In association, 2-IPMA and 3-IPMA did not show remarkable differences in MICs and MBCs values. However, using 2-IPMA together with 3-IPMA in the ratio 1:10, an interesting lower MIC and MBC (2048 mg/L and 4096 mg/L respectively) were observed against *Y. enterocolitica*, highlighting a possible synergistic effect of the two isomeric acids against this gram-negative strain. Interestingly, 3-IPMA showed a mild antioxidant activity by DPPH assay (EC_{50} = 3940 mg/L, 22.4 mM) higher than that of its isomer 2-IPMA (EC_{50} > 4800 mg/L). No toxicity of these compounds alone and in association was observed at all tested concentrations as evidenced by TB exclusion assay against human colorectal and liver cell lines. The mild antibacterial and mild antioxidant activities together with the good biocompatibility made 2- and 3-IPMA interesting candidates as preservatives for foods.

Conflict of Interests

The authors declare that no competing interests exist.

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