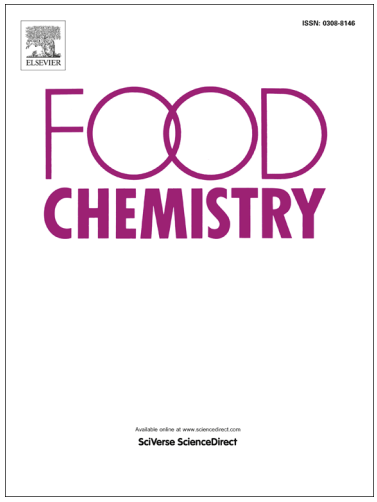

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Identification and quantification of new isomers of isopropyl-malic acid in wine by LC-IT and LC-Q-Orbitrap

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Abstract

Organic acids of wine comprise one of the main taste groups, that of sourness. Two isomeric compounds with molecular weight of 176 u have been identified in wine using two LC-MS systems, LC-IT (ion trap) and LC-Q-Orbitrap. The two isomers are organic acids, 3-isopropylmalic acid (3-IPMA) never identified in wines, and 2-isopropylmalic acid (2-IPMA), never quantified in wines. After the definitive identification against the authentic standards, an analytical method for their determination in wines was optimised and validated using the LC-IT platform. Linearity was verified in the range 5-320 mg L^{-1} (correlation coefficients higher than 0.9914) and the recoveries obtained spiking the samples at two fortification levels were higher than 86.7%, with RSDs (n=9) lower than 15.1%. Finally, the two compounds were quantified in ten red and white Italian wines, and average concentrations were determined at 1.78 mg L^{-1} (0.56-4.13) and 23.0 mg L^{-1} (6.7-41.6) of 3-IPMA and 2-IPMA, respectively.

Keywords: wine, 2-isopropylmalic acid, 3-isopropylmalic acid, LC-IT, LC-Q-Orbitrap, untargeted analyses, identification

Abbreviations

2-IPMA 2-isopropylmalic acid, 3-IPMA 3-isopropylmalic acid, DAD diode array detector, EIC Extract ion chromatogram, ESI electrospray ionization, FT Fourier transform, FWHM (full width at half maximum) LC liquid chromatography, LIT linear ion trap, LLE liquid–liquid extraction, LOD limit of detection, LOQ limit of quantification, MS mass spectrometry, MS/MS tandem mass spectrometry, PTFE polytetrafluoroethylene, RP reverse phase, RSD relative standard deviation, SPE solid phase extraction, TIC total ion chromatogram.

Chemical compounds studied in this article

2-IPMA (PubChem: CID 77), 3-IPMA (PubChem: CID 36)

1. Introduction

Wine is defined as an alcoholic beverage, which is produced by fermentation of fresh grapes or must. The organic acids in beverages are important in several respects. They

comprise one of the main taste groups, namely, that of sourness. All organic acids have this quality to some degree, but some have their own characteristic flavour, taste or aroma. For example, citric acid has a fresh acid flavour different from that of malic acid, while succinic acid has an unusual salty, bitter taste in addition to its sourness (Whiting, 1975). Oxo-acids (pyruvic and 2-oxoglutaric acids) bind SO_2, a widely used microbial inhibitor. Acids differ greatly in their susceptibility to microbiological attack: succinic acid is resistant anaerobically and aerobically while malic and citric acids are readily metabolized anaerobically with consequent flavor changes (Lamikanra, Inyang & Leong, 1995).

The quantitatively dominating acids of grapes are maleic, tartraric, and citric acids, the first two of which account for over 90% of the total acid content of grapes (Schreier & Jennings, 2013). Gas chromatographic methods have been applied for several decades for the analysis of acids in wines and grape juices (Fantozzi & Bergeret, 1973; Philip & Nelson, 1973). In 1974 Stahl et al. (Stahl, Laub & Woller, 1974) reported for the first time the determination of non-volatile acids in wines and fruit juices by liquid chromatography. The relative amounts of acids are also influenced by environmental factors such as temperature, light, and humidity. Changes in organic acid contents of grapes with fruit maturity and their distribution within mature berries have been determined by HPLC (Lamikanra, Inyang & Leong, 1995). Wine is also rich in phenolic compounds, that are important components as they affect organoleptic characteristics, such as colour, astringency and aroma (Kennedy, 2008). In addition, their antioxidant properties are thought to be associated with cardioprotective effects and other health benefits of moderate wine consumption (Cooper, Chopra & Thurnham, 2004). Some years ago, Ginjom et al. (2011) identified and quantified the individual phenolic compounds present in wine at different wine-making stages from crushing through to bottled and aged wine, produced in different Queensland (Australia) wineries. For this purpose, the phenolic components of

two red wines (Shiraz and Cabernet Sauvignon) were isolated by liquid–liquid extraction (LLE), purified by SPE and analyzed by HPLC-DAD-MS. Interestingly, they were not able to identify a compound (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. Previously, Monagas et al. reported the presence of a peak with similar characteristic (*m/z* 175 in the MS spectrum acquired in negative ion mode) in other wine types but its identity was not established (Monagas, Suarez, Gomez-Cordoves & Bartolome, 2005). Our group noticed this unknown peak by HPLC-DAD-MS performing a recent published study entitled "Further Highlighting on the Prevention of Oxidative Damage by Polyphenol-Rich Wine Extracts" (Salucci, Lucarini & Diamantini, 2017). Mass spectrometry is the most selective technique for the rapid qualitative and quantitative determination of known compounds. On the other side, for the identification of unknown compounds, the combination of Fourier Transform Orbitrap MS technology with a linear ion trap MS has been shown to give excellent results as it allowed identification and quantitation in untargeted and targeted analysis (Caprioli, Cahill & James, 2014a; Caprioli, Logrippo, Cahill & James, 2014b). Thus, the first aim of this work was to characterize the unidentified compound in wines with a nominal molecular mass of 176 u (Ginjom et al. 2011; Monagas et al. 2005) by using two LC-MS platforms, i.e. LC coupled to an ion trap detector (LC-IT) and LC coupled to a hybrid high-resolution mass analyser (LC-Q-Orbitrap). In the second part of this paper, thanks to identification of two isomers of isopropylmalic acid and availability of the authentic standards, for the first time these organic acids have been quantified in ten red and white wines.

2. Materials and Methods

2.1 Reagents and standards

The analytical standards of 2-IPMA (CAS Number 3237-44-3) and 3-IPMA (CAS Number 16048-89-8) were purchased from Sigma-Aldrich (Milano, Italy). The stock standard solution was prepared by dissolving 10 mg of each analyte in 10 mL of methanol and stored in a glass-stoppered bottle at 4°C in the dark. Standard working solutions, at various concentrations, were prepared daily by appropriate dilution of aliquots of the stock solutions in methanol.

HPLC-grade acetonitrile and methanol were purchased from Sigma-Aldrich (Milan, Italy), while HPLC-grade formic acid 99-100% was bought from J.T. Baker B.V. (Deventer, Holland). For sample preparation and chromatographic analysis, de-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) and wine characteristics are detailed in Table 2.

2.3 Sample preparation

50 mL wine samples were extracted three times with ethyl acetate (50 mL) following a previous published method (Salucci, Lucarini & Diamantini, 2017). The ethyl acetate extracts were pooled together prior to rotary evaporation at 30°C. This amount of samples (50 mL) and solvents (3 X 50 mL) can eventually be downscaled even if is important to minimize emulsion formation and to obtain good reproducibility among analysed samples. The residue was re-dissolved in 2.5 mL methanol, filtrated through a 0.2 µm membrane filter from Phenomenex (Bologna, Italy) and then directly injected into the LC-IT or LC-Q-Orbitrap systems.

2.4 LC-IT analysis

LC-IT studies were performed using an Agilent 1100 (Santa Clara, CA, USA) series instrument, made from an autosampler, a binary solvent pump, with a diode-array detector (DAD) and a mass spectrometer detector Trap SL (Bruker, Billerica, MA, USA) equipped with an electrospray ionization (ESI) source. The analyte separation was achieved on a Grace reversed phase (RP) (150 x 2.1 mm, 3 μm) LC column from Grace Davison Discovery Sciences (Columbia, MD, USA). The mobile phases were water with 0.1% formic acid (A) and acetonitrile (B) 95:5 v/v working in the gradient mode at a flow rate of 0.2 mL min-1. The solvent composition varied as follows: 0 min, 5% B; 2 min, 5% B; 2–4 min, 15% B; 4–12 min 30% B; 12–22 min, 60% B; 22–25 min 60% B; then the column was reconditioned. The column temperature was set at 35 °C and the injection volume was 2 μL. The ESI source was operating in negative ionization mode (ESI-). The optimization of the ion trap detector conditions was carried out by flow injection analysis (FIA) (1 μL of the individual standard solutions at 50 μg mL^{-1}). The parameters set for ESI were as follows: nebulizer gas (nitrogen) pressure, 50 psi; drying gas (nitrogen) flow rate, 9 mL min^{-1}; dry gas temperature, 325 °C; capillary voltage, 3500 V. Mass scan range was set in the interval of *m/z* 50–1000 in negative ionization mode.

2.5 LC-Q-Orbitrap analysis

The chromatographic separation was achieved using the same LC column and conditions (gradient and mobile phases) reported above. The LC-MS platform consisted of a Dionex Ultimate 3000 UHPLC system and a Q-Exactive Plus mass spectrometer equipped with a HESI II source (Thermo Scientific, San Jose, CA, USA). HESI II was operating in negative ionization mode setting the following parameters: capillary temperature 300 °C, vaporization temperature 320 °C, sheath gas flow 35, aux gas flow 15, source voltage 2.8 (KV), S-lens 50 (V). The mass range of full scan experiments was within *m/z* 100–400.

The data were acquired at 140,000 FWHM at *m/z* 200. The AGC representing the maximum capacity in C-trap was set at 5 x 10^5 ions for a maximum injection time of 600 ms. For t-MS^2 experiments the resolution was set at 140,000 FWHM (at *m/z* 200), the AGC target at 5 x 10^5, the injection time at 600 ms and the isolation window at 1.2 *m/z*. The stepped collision energy used were 10-20-30 NCE (normalized collision energy).

Results and discussion

3.1 Preliminary investigation by LC-IT

Preliminary experiments demonstrated that negative ion ionisation gave better results (in terms of intensity) with respect to the positive one (*Data not shown*). Figure 1 reports the total ion chromatogram (TIC) (a) and the extract ion chromatogram (EIC) of the ion at *m/z* 175 (b) of a wine sample extract and acquired in negative ionisation mode (ESI-). The TIC is quite rich and contains many peaks; on the other side, from the EIC (*m/z* 175), an abundant and broad peak at a retention time of 12.2 minutes is clearly visible (Figure 1b). In the full scan mass spectrum of this peak, the most abundant ion was at *m/z* 175 $[M-H]^-$ (Figure 1c); correspondingly, in the ESI(+) full scan spectrum, the most abundant ion species was at *m/z* 177 ($[M+H]^+$) (*Data not shown*). Thus, it is supposed that the unknown compound had a nominal molecular weight of 176 u. Reviewing the pertinent literature (Wojdyło, Samoticha, Nowicka & Chmielewska, 2018; Šeruga, Novak & Jakobek, 2018; Teixeira, Mateus, Freitas & Oliveira, 2018; Donato et al., 2018), we tried to understand if there were some polyphenols or typical wine substances with this molecular weight, but without any success. Thus we moved to perform MS^n studies. The MS^2 spectrum of the $[M-H]^-$ (*m/z* 175) (Figure 1d) shows a fragment ion at *m/z* 157 due to loss of water (*m/z* 18). In addition, there is also an intense signal at *m/z* 129, which correspond to the loss of formic acid (*m/z* 46) $[M-HCOOH-H]^-$, as well as the ion species at *m/z* 113 is due to the loss of CO_2 and H_2O $[M-CO_2-H_2O]^-$ from the precursor ion *m/z* 175. The ion at *m/z* 85

correspond to $[C_4H_5O_2]^-$ and, eventually, can be produced by the loss of CO_2 from the *m/z* 129. Observing in detail the MS^2 spectra (Figure 1d) all along the shape of the chromatographic peak at 12.2 min ((Figure 1b), it is evident that the ion species at *m/z* 73 is present only in the left side of the peak, whereas that at *m/z* 115 is detectable only in the right side, testifying the possible presence of two co-eluting compounds. The first ion species at *m/z* 73 may be produced by the loss of $C_5H_{10}O_2$ from the precursor ion (*m/z* 175), meanwhile the second one (*m/z* 115) by the loss of acetic acid $[M\text{-}CH_3COOH\text{-}H]^-$. Finally, even if the elucidation of the mass fragmentation pathways seems to be consistent, in order to obtain the definitive peak identification, further experiments were undertaken using a high resolution MS system, i.e. LC-Q-Orbitrap.

3.2 Identification of the unknown features by LC-Q-Orbitrap

The analysis by LC-Q-Orbitrap (t-MS^2) of a wine extract clearly confirmed the presence of two co-eluting compounds, the first characterised by the fragment at *m/z* 72.9914 (left side of the chromatographic peak), and the second one characterised by the fragment at *m/z* 115.0386 (right side of the chromatographic peak). The acquisition of the accurate mass supported the above reported hypothesis, that is the formation of these two ion species starting from the deprotonated molecule $[M\text{-}H]^-$, respectively, from the neutral loss of $C_5H_{10}O_2$ (*m/z* 72.9916) and acetic acid (*m/z* 115.0386). Raw data were then processed with Freestyle 1.1 software (Thermo Fisher Scientific, Waltham, MA, USA) performing identification by comparison with the mzCloud™ data (https://www.mzcloud.org). An excellent matc was obtained for the two isomeric organic acids, 3-IPMA and 2-IPMA, the first eluting in the left and the second in the right side of the LC-Q-Orbitrap chromatographic peak, respectively. The chemical structures of the two compounds are reported in Figure 1 Supplementary materials (Fig. S1). Examining the MS^2 spectra in

Figure 2a, the accurate mass of the precursor ion $[M-H]^-$ is *m/z* 175.0602. This measured value minus the exact mass of the deprotonated isopropylmalic acids ($[C_7H_{11}O_5]^-$ at *m/z* 175.0612) give an error equal to -5.7 ppm, corroborating the compound identity. Analogously, the accurate masses of the losses hypothesized to explain the characteristic fragment ion at *m/z* 72.9914 and at *m/z* 115.0386 were *m/z* 102.0688 (*m/z* 175.0602 - *m/z* 72.9914) and *m/z* 60.0216 (*m/z* 175.0602 - *m/z* 115.0386), respectively (Figure 2a). These values fully confirmed, in the first case, the loss of a 3-metylbutanoic acid residue ($C_5H_{10}O_2$, mass error +7.1 ppm) for 3-IPMA and, in the second one, of an acetic acid residue ($C_2H_4O_2$, mass error +7.8 ppm) for 2-IPMA. The comparison between the 3-IPMA and 2-IPMA spectra from the library (at the top) and from the HRMS experiments (at the bottom) conducted in the current study, is shown in Figure 2b. In the middle, the "difference spectrum" highlights that there are negligible differences among the experimental spectrum (called "query") and that coming from the *m/z*Cloud library.

3.3 Confirmation of the identified compounds with authentic standards

After the identification study carried out with the LC-Q-Exactive Plus platform, the authentic standards of 2-IPMA (CAS Number 3237-44-3) and 3-IPMA (CAS Number 16048-89-8) were purchased from Sigma-Aldrich and analysed using the LC-IT (ESI-). As expected, in the chromatogram two co-eluting peaks were observed at 11.7 min (3-IPMA) and at 12.4 min (2-IPMA). In Figure 3 the LC–IT spectra recorded from the standard solutions of 2-IPMA (a) and 3-IPMA (b) are shown. The MS^2 spectra of the authentic standards (amplitude 0.9) confirmed the data already obtained: 3-IPMA displayed its own peculiar ion species at *m/z* 73, whereas 2-IPMA at *m/z* 115 (Figure 3).

All the other fragments (*m/z* 157, *m/z* 129, *m/z* 113, *m/z 85*) were shared by both isomers. The first peculiar fragment (*m/z* 73) is typical of 3-IPMA and in fact is produced by the loss of $[C_5H_{10}O_2]$ (*m/z* 102), giving the ion $[C_2HO_3]^-$ at *m/z* 73; on the other hand, the second peculiar one (*m/z* 115) is produced by the loss of acetic acid $[M-CH_3COOH-H]^-$ from 2-IPMA (Figure S1).

In Figure 3 it is possible also to appreciate the differences of the ion abundances between the two compounds. In addition, a real red wine extract was injected in the ion trap apparatus (MS/MS) with amplitude 0.9.

In Figure 4 the overlapped chromatograms are reported showing the peaks obtained extracting the specific ion fragments (i.e. *m/z* 115 or *m/z* 73) from the standard solutions of the authentic compounds and from the red wine. In the wine both compounds were detected: 3-IPMA at a retention time of 11.7 min and 2-IPMA at a retention time of 12.4 min.

3.4 Method validation of the LC-IT method

After the definitive identification against the authentic standards, an analytical method for their quantification in wines was validated using the LC-IT platform. The investigated performance characteristics of the developed method were linearity, limit of detection (LOD), limit of quantification (LOQs), recovery, intraday and interday precision and matrix effect (as signal suppression/enhancement %) (Table 1).

Linearity was tested by injecting 6 different concentrations of standard mixtures of the analytes in solvent (Table 1) from 5 to 320 mg L^{-1} (corresponding to a range of 0.25-16 mg L^{-1} in matrix). Calibration curves (concentrations versus peak areas) were determined by least-squares regression analysis obtaining correlation coefficients (R^2) higher than 0.9914. The reproducibility of the chromatographic retention times was examined five times over a five day period (*n*=25) obtaining high stability (RSD < 1%).

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 0.2 and 0.5 mg L^{-1}, for both compounds (Table 1). The mean recoveries obtained spiking a wine sample thrice in three different days at 5 mg L^{-1} were 86.7 (2-IPMA) and 90.1% (3-IPMA) with RSDs (*n*=9) equal to 12.1% and 15.1%, respectively (Table 1). The mean recoveries obtained spiking a wine sample thrice in three different days at level of 50 mg L^{-1} were from 90.7 (2-IPMA) and 93.2% (3-IPMA) with RSDs (*n*=9) equal to 8.4% and 8.9%, respectively (Table 1).

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^{-1}. 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):

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

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 1).

In the following paragraph, the quantification of the two analyte in wine samples have been reported taken into account ME studies.

3.5 Quantification of 2-IPMA and 3-IPMA in Italian wines

The validated LC-IT method was used to analyse ten Italian wine samples, specifically five red and five white wines. The two analytes were found in all samples. In Table 2 the results were reported in detail; each wine sample was analysed in triplicate. Concerning red wines, the contents of 2-IPMA ranged from 18.9 mg L^{-1} (Montepulciano) to 41.6 mg L^{-1} (Merlot). Also "Primitivo" displayed a conspicuous content of 2-IPMA (30.1 mg L^{-1}). On the other hand, 3-IPMA was found in lower concentration ranging from 1.43 mg L^{-1} (Montepulciano) to 4.13 mg L^{-1} (Merlot). The average content of 2-IPMA in the five red wines was 28.4 mg L^{-1}, whereas that of 3-

IPMA was 2.23 mg L^{-1}. Concerning the white wines, the contents of 2-IPMA ranged from 6.7 mg L^{-1} (Primofiore) to 27.8 mg L^{-1} (Verdicchio di Matelica), meanwhile the content of 3-IPMA was from 0.56 mg L^{-1} (Primofiore) to 2.42 mg L^{-1} (Verdicchio di Matelica). The average contents of 2-IPMA and 3-IPMA in white wines were 17.5 mg L^{-1} and 1.32 mg L^{-1}, respectively.

From literature, α-isopropylmalate is reported to be a leucine biosynthesis intermediate in yeast. In *Saccharomyces cerevisiae*, α-isopropylmalate, which is produced in mitochondria, is exported to the cytosol where it is required for leucine biosynthesis (Marobbio, Giannuzzi, Paradies, Pierri, & Palmieri, 2008). Yeast cells naturally secret this compound into their surrounding. It is thought that 2-IPMA secretion chelates aluminium ions and prevents them from entering cells, resulting in aluminium tolerance (Suzuki, Tamura, Nakanishi, Tashiro, Nishizawa, & Yoshimura, 2007) and reducing aluminum toxicity to the yeast cell.

According to the possible implication of 2-IPMA in the flavour of wines, really scant information is available in literature. Skogerson et al. (2009) determined the metabolite profiles of white wines, including Chardonnay, Pinot gris, Riesling, Sauvignon blanc, and Viognier varieties, by using gas chromatography-coupled time-of-flight mass spectrometry (GC-TOF-MS). 2-Isopropylmalate detected appeared to have higher mean area (semi-quantitative approach) in the low-wine body classification group (Skogerson, Runnebaum, Wohlgemuth, De Ropp, Heymann, & Fiehn, 2009).

No data are available in literature for 3-IPMA, that has been identify and quantify in wine for the first time in the current study.

4. Conclusions

Organic acids of wine are important as they comprise one of the main taste groups, namely, that of sourness. Two organic acids (nominal molecular weight of 176 u) using two LC-MS platforms such as LC-IT and LC-Q-Orbitrap have been identified and quantified in wines. Thanks to the combined information obtained from the ionization behaviour, fragmentation experiments and accurate mass acquisition, it was possible to establish that they were the two isomeric forms of isopropylmalic acid (2-IPMA and 3-IPMA). The definitive confirmation was carried purchasing authentic standards.

A quantitative analytical method by using the LC-IT technique was then developed and validated. Although the chromatographic peaks of the two analytes were not well separated, two peculiar ion species, *m/z* 115 for 2-IPMA and *m/z* 73 for 3-IPMA, were found allowing to measure each compound separately by extracting their characteristic fragments. These newly identified compounds were quantified in ten red and white Italian wines highlighting that 2-IPMA was about ten-fold more concentrated than 3-IPMA with average levels of 23.0 mg L^{-1} and 1.78 mg L^{-1}, respectively.

The future perspective is to increase the available data about the IPMA contents in wines also evaluating whether they could depend on year of production, colour, variety, etc.

In this sense, it would be of interest to downscale the extraction method in such a way to characterize a larger number of samples.

Conflict of Interests

The authors declare that no competing interests exist.

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Figure captions

Figure 1.

LC–IT chromatograms and spectra: a) Total ion chromatogram (TIC), b) Extract ion chromatogram (EIC) of ion species at *m/z* 175; c) ESI full scan mass spectrum and d) MS/MS spectrum (*m/z* 175 base peak).

Figure 2.

Figure 2a. LC-Q-Orbitrap spectra of a wine extract: A) MS2 spectrum of the first eluting compound (3-IPMA, Retention time 7.47 min); B) MS2 spectrum of the second eluting compound (2-IPMA, Retention time 8.10 min). Figure 2b. Left) MS^2 spectrum of 3-IPMA from m/zCloud database (A): MS^2 experimental spectrum of 3-IPMA (C). Right: MS^2 spectrum of 2-IPMA from m/zCloud database (A); MS^2 experimental spectrum of 2-IPMA (C). In the middle (B) the difference between the experimental and the m/zCloud spectra.

Figure 3.

LC–IT MS^2 spectra of 2-IPMA (a) and 3-IPMA (b) recorded from individual solution of the authentic standards with the same amplitude.

Figure 4.

LC-IT MS^2 chromatogram showing the overlapped EICs of: a) *m/z* 115 ion from the wine sample, b) *m/z* 115 ion from the standard of 2-IPMA (0.16 mg/mL), c) *m/z* 73 ion from the wine sample, d) *m/z* 73 ion from the standard of 3-IPMA (0.16 mg/mL).

Table 1. Validation data: linearity range, LODs, LOQs, recoveries (Rec%) and repeatability (RSD%) evaluated at two fortification levels (n=9) and matrix effect as signal suppression/enhancement (SSE %).

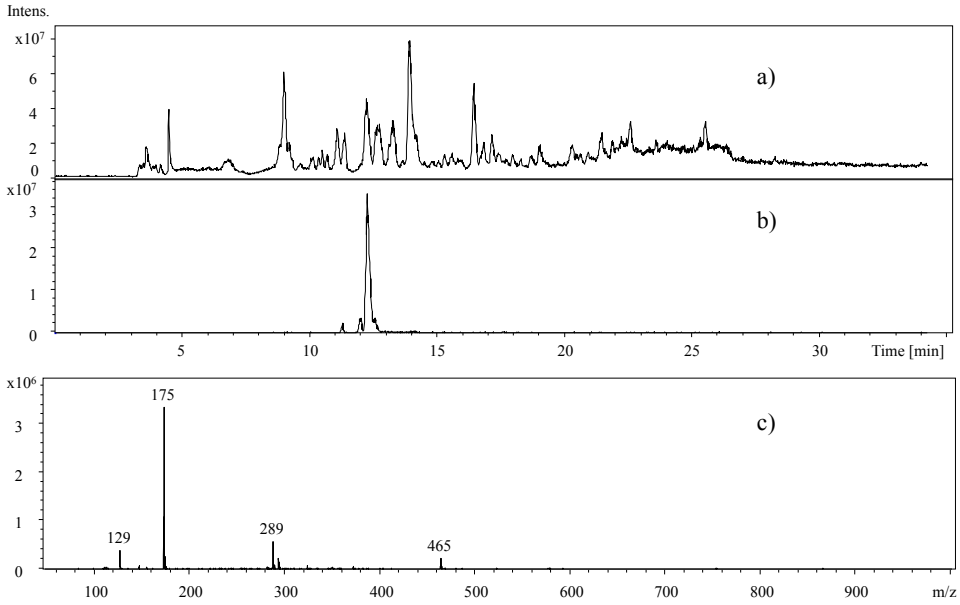
Analyte	Linearity Range (mg L^{-1})	R^2	LOD[a] (mg L^{-})	LOQ[b] (mg L^{-1})	Rec % (5 mg L^{-1})	RSD% (5 mg L^{-1})	Rec % (50 mg L^{-1})	RSD% (50 mg L^{-1})	Matrix effect (SSE %)
3-IPMA	5-320	0.9914	0.2	0.5	90.1	15.1	93.2	8.9	59
2-IPMA	5-320	0.9931	0.2	0.5	86.7	12.1	90.7	8.4	46

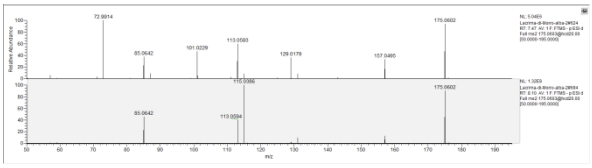
Table 2. Quantification of 3-IPMA and 2-IPMA (mg L^{-1}) in ten Italian wine samples (± standard deviation, *n*=3).

N°	Year	Colour	Type	3-IPMA (mg L^{-1})	2-IPMA (mg L^{-1})
1	2010	Red	Montepulciano	1.43 ± 0.09	18.9 ± 0.8
2	2016	Red	Primitivo	1.87 ± 0.14	30.1 ± 0.8
3	2016	Red	Merlot	4.13 ± 0.08	41.6 ± 1.1
4	2016	Red	Lacrima	1.85 ± 0.03	28.1 ± 0.7
5	2017	Red	Lacrima	1.87 ± 0.12	23.3 ± 1.3
Average content red wines				2.23	28.4
6	2017	White	Verdicchio di Matelica	2.42 ± 0.08	27.8 ± 0.5
7	2017	White	Verdicchio dei Castelli di Jesi	0.9 ± 0.06	10.4 ± 0.6
8	2017	White	Valdobbiadene	1.16 ± 0.07	18.5 ± 0.8
9	2017	White	Muller Thurgau	1.57 ± 0.15	24.3 ± 1.1
10	2017	White	Primofiore	0.56 ± 0.07	6.7 ± 0.2
Average content white wines				1.32	17.5
Average content of all samples				1.78	23.0

Highlights

- 2- and 3-isopropyl malic acid were identified and quantified in wine
- Combined MS techniques were applied to achieve definitive identification
- An analytical method was developed and validated to quantify both isomers
- The average contents of the two acids were assessed in a group of ten wines

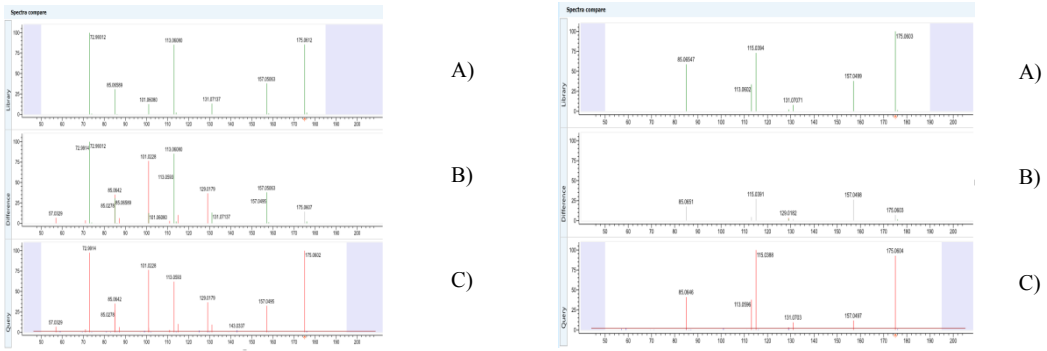




A)

B)

Figure 2a. LC-Q-Orbitrap spectra of a wine extract: A) MS^2 spectrum of the first eluting compound (3-IPMA, Retention time 7.47 min); B) MS^2 spectrum of the second eluting compound (2-IPMA, Retention time 8.10 min)



A)

B)

C)

A)

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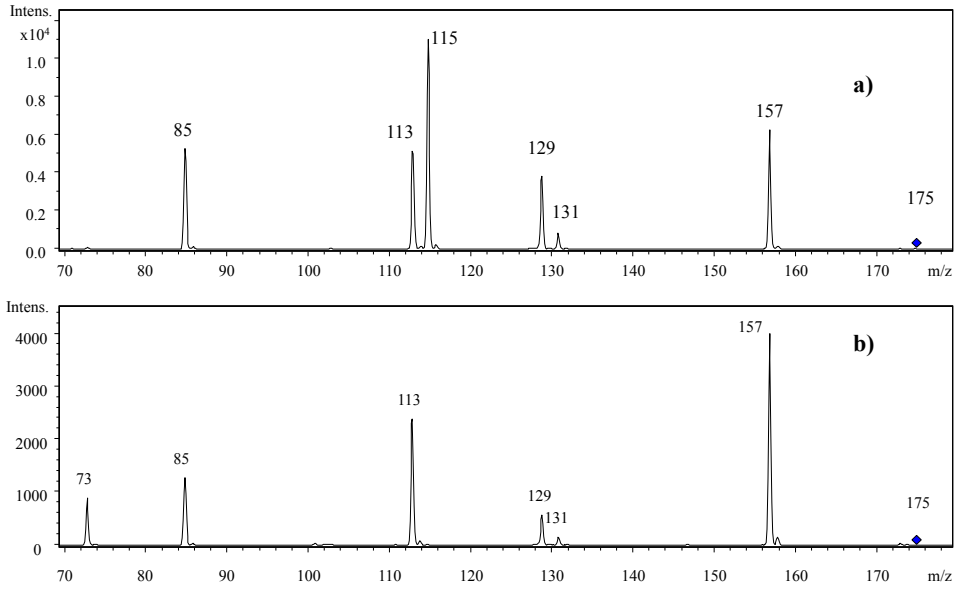
Figure 2b. Left) MS^2 spectrum of 3-IPMA from m/zCloud database (A): MS^2 experimental spectrum of 3-IPMA (C). Right: MS^2 spectrum of 2-IPMA from m/zCloud database (A); MS^2 experimental spectrum of 2-IPMA (C). In the middle (B) the difference between the experimental and the m/zCloud spectra.



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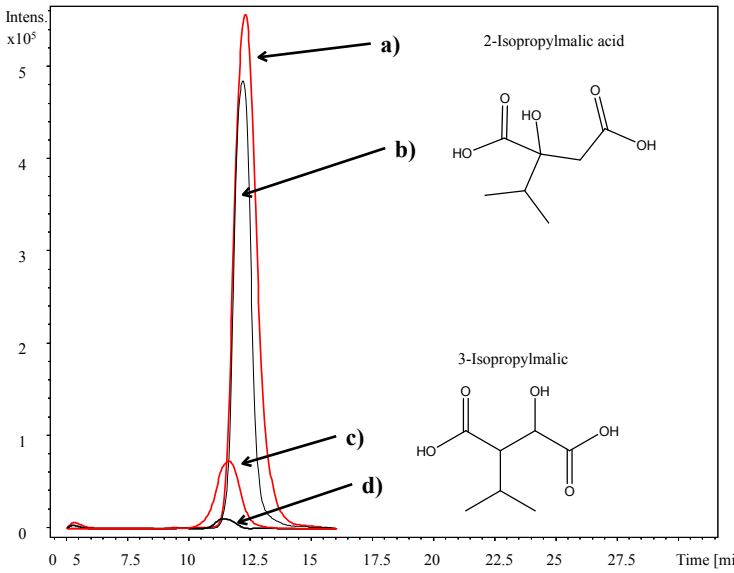

Figure 4. LC-IT MS^2 chromatogram showing the overlapped EICs of: a) *m/z* 115 ion from the wine sample, b) *m/z* 115 ion from the standard of 2-IPMA (0.16 mg/mL, ret time 12.4 min.), c) *m/z* 73 ion from the wine sample, d) *m/z* 73 ion from the standard of 3-IPMA (0.16 mg/mL, ret time 11.7 min.).