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Evaluation of antioxidant supplementation in must on the development and potential reduction of different compounds involved in atypical ageing of wine using HPLC-HRMS

Tiziana Nardin, Tomas Roman, Susanne Dekker, Giorgio Nicolini, Francesco Thei, Barbara Masina, Roberto Larcher *

Technology Transfer Centre, Fondazione Edmund Mach, Via E. Mach 1, 38010 San Michele All'Adige, Italy

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ABSTRACT

Atypical aging of white wines (ATA) is an off-flavour characterised by rapid loss of fruity aromas and the development of unpleasant odours.

This study aims to evaluate the effectiveness of different oenological adjuvants (4-ethylcatechol, 4-methylcatechol, gentisic acid, hydroxytyrosol, ascorbic acid, glutathione, ellagic acid, gallic acid, galla tannin, ellagic tannin, quebracho, grape tannins) added to musts before fermentation for preventing the possible development of ATA. High resolution mass spectrometry made it possible to quantify and qualify free and conjugated IAA in wine at the end of the fermentation and subsequently 2-aminoacetophenone (AAP) was quantified after the force ageing period (6 days at 40 °C). Ascorbic acid was confirmed as the most appropriate antioxidant adjuvant which can be used for ATA defect prevention. With an almost comparable effect, gallotannin addition prevented AAP production to exceed 1 μg/L. A predicted model (ANCOVA) indicated that over 80% of the variability of potential AAP formation in wines was explained by the amount of precursors, grape variety and antioxidant treatment. Moreover, a suspect screening approach made it possible to study the kinetic formation and the consumption of the reaction metabolites formed during the oxidative degradation of IAA leading to AAP.

1. Introduction

Atypical aging (ATA) is an off-flavour that can occur in young white wines and result in an early loss of varietal aroma and the development of scents of mothball, wet mop, sweat, acacia blossom, soap, shoe polish or wax taints (Fan et al., 2007; Rapp et al., 1993). The first reports on German Riesling wines (Sponholz & Hühn, 1996a) described the identification of this defect and revealed the presence of ATA in various wines produced all over the world (V. Schneider, 2013). Although a large part of the mechanisms and factors involved in ATA are still unclear, 2-aminoacetophenone (AAP) was found to be the compound primarily responsible for this aroma defect. AAP has a sensory threshold between 0.5 and 10.5 μg/L, depending on the complexity of wines (Fan et al., 2007; Gessner et al., 1995; Rapp et al., 1993; Hoenicke, Simat, et al., 2002; Perry & Hayes, 2016). The compound 3-indole-acetic acid (IAA), being the primary auxin in plants, has been defined as the most prominent among the different possible precursors of AAP. Other possible precursors have been suggested by previous researchers, including skatole (SKA), indole or 1,1,6-trimethyl-1,2-dihydronaphthalene (Gessner et al., 1995; Nikfardjam et al., 2005; Simat et al., 2004; Sponholz & Hühn, 1996b), while conflicting opinions are reported for kynurenine (KYN) (Dollmann et al., 1996; Hoenicke, Simat, et al., 2002).

Two main pathways have been proposed for the biosynthesis of IAA in vines: the TRP-dependent and TRP-independent pathway. As suggested from previous studies, TRP-independent biosynthesis involves the precursors indole or indole-3-glycerol phosphate, although a genetic basis has not been defined yet (Facchini et al., 2000; Zhang et al., 2008). On the other hand, four distinct pathways have been proposed for the TRP-dependent biosynthesis: indole-3-acetamide (IAM), indole-3-pyruvic acid (IPA), tryptamine (TAM) and indole-3-acetaldoxime (IAOx) with indole-3-acetonitrile (IAN) as intermediate metabolite (Mano et al., 2010; Pollmann et al., 2006).

Studies revealed the presence of auxin compounds in various plant materials. IAA esters bound to *myo*-inositol and other sugars, such as arabinose and galactose were found in Corn Kernels of *Zea Mais* (Nicholls, 1966; Shantz & Steward, 1957), esters with glycoproteins in *Avena Sativa* (Percival & Bandurski, 1976), aspartate and glutamate conjugates

* Corresponding author.
E-mail address: roberto.larcher@fmach.it (R. Larcher).

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Abbreviations

2-aminoacetophenone AAP
3-(2-formylaminophenyl)-3-oxopropinoic acid FAPOP
3-indole-acetic acid IAA
3-indolacetic acid-hexoside IAA-hexoside;
ascorbic acid Asc
atypical aging ATA
diammonium hydrogen phosphate DAP
ellagic tannin ET
formyl-2-aminoacetophenone FAP
galla tannin GaT
glutathione GSH
grape tannin GrT
indole-3-acetaldoxime IAOx
indole-3-acetamide IAM
indole-3-acetonitrile IAN
indole-3-lactic acid ILA
indole-3-pyruvic acid IPA
indole-acetic acid-2-sulfonate IAA-SO_3H
Johanniter Jn
methyl-indole-3-acetate me-IAA
oxidized indole-acetic acid Ox-IAA
N-(3-indolylacetyl)-DL-aspartic acid IAA-Asp;
N-(3-indolylacetyl)-L-alanine IAA-Ala
Pinot blanc PB
Pinot gris PG
Riesling Rs
skatole SKA
tryptamine TAM
tryptophan TRP
tryptophol TRH

in soybean callus (Arjmand et al., 1978) and IAA-N-glucosides in Scots Pine Seedlings (Ljung et al., 2001). In grape must, over 90% of the total IAA is bound either as ester or amide conjugate, while the free form is present at very low concentration (Hoenicke et al., 2001; Simat et al., 2004). However, yeast activity during alcoholic fermentation can facilitate the cleavage of the bonds releasing free IAA (MihaljevićZulj et al., 2015) or induce production of free IAA from tryptophan (TRP) (Liu et al., 2016). Two different pathways of IAA formation have been reported: the IPA pathway resulting from the TRP yeast's metabolism, with indole-3-lactic acid (ILA) as an intermediate metabolite, and the tryptophol (TRH) pathway, starting from the amino acid TRP (Álvarez-Fernández et al., 2019).

As previously described by Hoenicke, Simat, et al. (2002), free IAA can also result in the formation of other conjugates during the wine-making process, for example the sulfonated form of IAA (IAA-SO_3H). This compound is a possible intermediate for the formation of AAP and formerly identified by Arapitsas et al. (2018) in white and sparkling wines.

During alcoholic fermentation and wine aging, IAA degradation starts with cleavage of the pyrrole ring to form 3-(2-formylaminophenyl)-3-oxopropinoic acid (FAPOP). This reaction is initiated by a superoxide radical from which a large part is released during the co-oxidation of sulphite to sulfate (Christoph et al., 1998). Subsequently, the decarboxylation of FAPOP takes place, resulting in the formation of formyl-2-aminoacetophenone (FAP) and finally AAP, or alternatively, oxidized indole-acetic acid (Ox-IAA) (Hoenicke, Simat, et al., 2002). ATA normally does not appear in red wines, probably due to the high antioxidant capacity from naturally present grape phenols. This led to the hypothesis that increasing the antioxidant capacity of white wines could improve the protection against ATA. Moreover, literature studies reported a negative correlation between antioxidant concentration and ATA (Hoenicke, Simat, et al., 2002) and a slight negative correlation with AAP (Linsenmeier & Löhnertz, 2016). Besides the pre-fermentative strategies, including properly selected pressing techniques (Roman et al., 2020) and sufficient clarification of the must (Köhler et al., 1996), antioxidant addition is the only effective post-fermentation treatment for ATA prevention known so far. Previous studies have not shown clear effects from tannin supplementation (100 mg/L) and the addition of glutathione (GSH, 10–150 mg/L) was only effective in some wines (Volker Dubourdieu & Lavigne, 2004; Schneider, 2014). In contrast, ascorbic acid (Asc, 75 and 150 mg/L) has been shown to successfully prevent the formation of AAP (Rauhut et al., 2003). However, undesired pro-oxidative effects of Asc have been observed, among which the formation of hydrogen peroxide and oxygen radicals (Bradshaw et al., 2003). Nevertheless, Asc addition may provide some complementary protection of fruity aromas against oxidative aging when used in conjunction with adequate levels of free SO_2 (Volker Schneider, 2016). Therefore, Asc can display either pro-oxidative or reductive effects, depending on the age and oxidation state of the wine (Peng et al., 1998).

In this study the possible prevention of ATA development was explored by evaluating several aspects related to the ATA defect, including the pre-fermentative addition of some phenolic compounds and oenological adjuvants (4-ethylcatechol, 4-methylcatechol, gentisic acid, hydroxytyrosol, ASC, GSH, ellagic acid, gallic acid, galla tannin (GaT), ellagic tannin (ET), quebracho, grape tannins (GrT)). Protective redox potentials (Larcher et al., 2008), the natural concentration in grapes and wines (Barnaba et al., 2015, 2017) and the usual technological doses of these adjuvants were taken into account to define the treatments. High performance liquid chromatography coupled with high resolution mass spectrometry (HPLC-HRMS) was used to evaluate the AAP precursor content in wines after each of the different adjuvant treatments and subsequently the AAP content after an accelerated ageing. In order to gain more insight in the potential formation of AAP, different precursors were quantified and qualified through both a targeted approach and a suspect screening approach by adapting the analytical method proposed by Roman et al. (2020). Moreover, the formation and consumption kinetics of the reaction metabolites from oxidative degradation of the most prominent IAA were evaluated for the first time. ANCOVA modelling was used to predict the possible AAP production considering grape varieties, treatments and IAA content in young wine as known variables.

2. Materials and methods

2.1. Chemicals and reagents

Liquid chromatography-mass spectrometry (LC-MS)-grade acetonitrile (ACN), LC-MS grade methanol (MeOH), ethanol (>99.8%; EtOH) and MS grade formic acid (FA, 98%) were purchased from Fluka. 4-ethylcatechol (≥98%), 4-methylcatechol (≥95%), AAP (98%), Asc (≥99%), ellagic acid (≥96%), gallic acid (≥97.5%), gentisic acid (≥98%), hydroxytyrosol (≥98%), IAA (99%), IAM (98%), IAN (98%), ILA (99%), IPA (>97%), KYN (>98%), L-glutathione reduced (GSH, >98%), methyl-indole-3-acetate (98%; me-IAA), N-(3-indolylacetyl)-L-alanine (98%; IAA-Ala), N-(3-indolylacetyl)-DL-aspartic acid (98%; IAA-Asp), SKA (98%), TAM (>97%), TRH (≥98%), TRP (≥98%) and potassium disulfite (≥98%; $K_2S_2O_5$), diammonium hydrogen phosphate (≥98%; DAP), hydrogen peroxide solution (30%, w/w) were purchased from Sigma-Aldrich. Deionized water (H_2O) was produced with an Arium Pro Lab Water System (Sartorius AG). A standard mix of Ultramark 1621, *n*-butylamine, caffeine, methionine-arginine-phenylalanine-alanine

(MRFA) (Pierce® ESI Positive Ion Calibration Solution, Rockford, IL, USA) was used for positive mass calibration while a standard mix of Ultramark 1621, formic acid, sodium dodecyl sulfate and sodium taurocholate was used for negative mass calibration. *Saccharomyces cerevisiae* yeast (Maurivin AWRI 796), ET, GrT, GaT, quebracho, grape skin and grape seed tannin were sourced from the Fondazione Edmund Mach experimental microwinery.

2.2. Analytical method

ATA precursors and AAP were chromatographically separated using Thermo Ultimate R3000 ultra-high performance liquid chromatography (UHPLC) (Thermo Scientific) coupled with a Q-Exactive hybrid quadrupole-orbitrap mass spectrometer (HQOMS, Thermo Scientific) equipped with a heated electrospray ionization (HESI-II) interface working in positive and negative ionization. Adapting the method of Roman et al. (2020), a chromatographic column Raptor Biphenyl 3 × 150 mm (2.7 μm particle size, Restek®, PA, USA) with a ternary mobile phase containing 1% of FA 2%, 20% of ACN and 79% of H_20 at 0.3 mL min^{-1} was used. Mass spectra were acquired with a full MS-data dependent MS/MS experiment (full MS–dd MS/MS). Mass resolution was set at 70,000 full width at half-maximum (FWHM, calculated for m/z 200, 1.5 Hz) for full MS spectra and at 17,500 FWHM (12 Hz) for dd-MS2. The scan range was m/z 100–500 for full MS mode and normalised collision energy (NCE), set at 30 arbitrary units. The HESI source was set as follows: spray voltage, 3 kV for positive ionization and 2.50 kV for negative ionization; sheath gas flow rate at 40 arbitrary units; auxiliary gas flow rate at 20 arbitrary units; capillary temperature at 330 °C; capillary gas heater temperature at 350 °C.

The characteristics of the quantitative method were studied using the 14 pure standards. Precursor ion detected in the extracted ion chromatograms (EICs), corresponding to the protonated molecule $[M+H]^+$, was used for the quantification. Matching of m/z values with a mass tolerance <5 ppm (SANCO/12571/2013), RTs and dd-MS/MS spectra were used for the qualification. The linearity range was evaluated considering a matrix-matched calibration curve obtained by plotting the peak area of quantifier ion versus the nominal concentration of 10 increasing levels from 0.05 to 500 μg/L (0.1–5000 μg/L for TRP), each replicated with 4 different injections and 7 increasing levels from 0.005 to 2 μg/L for AAP. Both limits of detection (LOD) and quantification (LOQ) were established according to EURACHEM (EURACHEM 2014). Method precision was estimated as the relative standard deviation (RSD %) of 10 analytical replicates of a spiked must sample, while trueness was expressed as the mean recovery (%) of 3 different must samples spiked with 100 μg/L for each compound with the exception of TRP spiked with 1000 μg/L and AAP spiked with 1 μg/L.

For the qualitative method, a suspect-screening approach was performed using the *in-house* prepared standard. The EICs corresponding to the exact mass of the deprotonated molecule $[M+H]^-$ (IAA-SO_3H) and protonated molecule $[M+H]^+$ (radical cation, FAP and Ox-IAA) were used to evaluate the correct RT of the compounds and the relative dd-MS/MS spectra for fragmentation study. The IAA-hexoside RT was studied with a full mass/all ion fragmentation/NL data dependent-MS2 (Full MS/AIF/NL dd-MS2) experiment in positive ion mode (Larcher & Nardin, 2019).

2.3. Standard preparation

Single solutions of pure standards were prepared in a glass flask by dissolving the 13 precursors in aqueous methanol solution (50:50, v/v) at a concentration of 100 mg/L each. A mix precursor solution was prepared combining the single solution with a final concentration of 5 mg/L each (50 mg/L for TRP). AAP standard was prepared at a concentration of 50 in ACN and then diluted to obtain a final concentration of 5 μg/L in aqueous methanol solution (50:50, v/v). The precursor mix and the AAP solutions were used to prepare the points of two different wine matrix-matched calibrations.

According to Arapitsas et al. (2018), a qualitative *in-house* prepared standard of indole-acetic acid-2-sulfonate (IAA-SO_3H) was made dissolving 27 mg of IAA in 2 mL of ethanol, and slowly pouring this solution into a $K_2S_2O_5$ solution (300 mg in 5 mL H_2O) under gentle stirring. The solution was left at 30 °C for 14 days and diluted 10 times with water before the HPLC-HRMS analysis.

To evaluate the retention time (RT) of the metabolites that were produced during the oxidative chemical reaction from IAA to AAP (radical cation, FAP and FAPOP) and the alternative reaction by-product Ox-IAA, a water solution of 100 μg/L of IAA were prepared directly in a HPLC vial and added with 10 μL of hydrogen peroxide solution. The vial was placed in the autosampler at 40 °C and injected into the HPLC-HRMS system every 10 h per 2 days to monitor the compound formation.

2.4. Evaluation of antioxidant effect: in-vitro test

Eight single compounds (4-ethylcatechol, 4-methylcatechol, Asc, ellagic acid, gallic acid, gentisic acid, GSH, hydroxytyrosol; dose of 0.125 g/L each) and 4 natural tannins (ellagic, quebracho, seed, and skin; 0.5 g/L each) were added separately in vials with 45 mL of 3 different musts (Pinot gris, PG; Sauvignon blanc, SB; Müller Thurgau, MT). The musts were inoculated with AWR yeast (20 g/hl) and DAP (500 mg/L). The fermentation was monitored evaluating the sugar content using the OIV enzymatic method (Glucose and fructose; OIV-MA-AS311-02) ("International Organisation of Vine and Wine (OIV)," 2021) and when reached the end the wine samples were immediately added with 50 mg/L $K_2S_2O_5$. The samples were divided in two HPLC vials: one was stored in the fridge at 4 °C and the other was subjected to a heat treatment (40 °C, 48 h) in order to accelerate the reaction processes. Subsequently, all the vials were analysed for the quantification of AAP.

2.5. Evaluation of antioxidant effect: microwinery sample preparation

Five different lots of musts, from grapes harvested in Trentino (Italy) in 2018 (n = 2 Riesling, Rs1 and Rs2; 1 Pinot gris, PG; 1 Pinot blanc, PB; 1 Johanniter, Jn) were prepared in 12 bottles (0.5 L) for each must and added with TRP (10 mg/L) and IAA (25 μg/L). The adjuvants (Asc, GSH, ET, GrT and GaT) were prepared separately in ultrapure water: EtOH (90:10 v/v). The antioxidant treatments were carried out in triplicate at the following concentrations: Asc, 100 mg/L; GSH, 20 mg/L; ET, GrT, and GaT, 250 mg/L; The initial concentration of the antioxidant solution was established so that this final concentration could be achieved adding 2.5 mL (antioxidant) to 0.5 L of must. Ethanol was needed to dissolve the tannin and to keep the equivalent matrix conditions as the other treatments, Ctr was added with 2.5 mL of ultrapure water: EtOH (90:10 v/v). Each bottle was then inoculated with a *Saccharomyces cerevisiae* yeast (20 g/hl; Maurivin AWRI 796) and after 6 days with 500 mg/L of DAP. The alcoholic fermentation was maintained at 18–20 °C and the sugar consumption by yeast was monitored by regular measurements of the must weight after standardisation with the OIV-MA-AS311-02 method. Finally, as soon as a sugar content <10 g/L was reached, the wines were added with 50 mg/L of $K_2S_2O_5$ and stored at 10 °C.

For each bottle, 3 samples were taken and filtered (0.45 μm PTFE filters) directly into the HPLC vial. Of the 3 replicas, one was placed in the fridge to keep the sample stable (4 °C, T_0) and the other two were placed in an oven at 40 °C to mimic an accelerated wine ageing. One aliquot was removed after 3 days (T_3) and the second one after 6 days (T_6) and placed in the fridge. The samples were analysed all together using UHPLC-HRMS.

2.6. Study of IAA, IAA-conjugates and metabolites evolution during wine aging

A PG must from the Fondazione Edmund Mach micro winery was

added with 25 μg/L of IAA and 10 mg/L of TRP and divided in 6 aliquots of which three were further added with 100 mg/L of Asc. The must samples were subjected to a fermentation (DAP, 500 mg/L; yeast Maurivin AWRI 796, 20 g/L; 18–20 °C; final sugar content <10 g/L) and 50 mL of the produced wines were collected in 6 falcons. The samples were added with 50 mg/L of $K_2S_2O_5$ and kept in an oven at 40 °C for a prolonged ageing of 25 days and samples were taken after 1, 2, 3, 6, 9, 14, 22 and 25 days. All the collected samples were stored in the fridge at 4 °C before the HPLC-HRMS analysis. The content of the screening compounds was extracted as signal (area, count*min) and then normalised to 100%. The evaluation was done on the average of three analytical replicates.

2.7. Statistical data evaluation

Data analysis was performed on the concentration for targeted compounds and on the ionization intensity (area, count*min) normalised with the peak area of the total ion current (TIC; Draper et al., 2013) for suspect-screening compounds using XLSTAT 2020 software (Addinsoft). A Friedman's test ($p < 0.05$) was used for comparing the different AAP precursor distribution in young wines and the AAP production during the accelerating aging. An Analysis of Covariance (ANCOVA) modelling was carried out to predict the final content of AAP depending on the different varieties, treatments and IAA content in young wines.

3. Results and discussion

3.1. Analytical method

The method reported by Roman et al. (2020) was used to quantify 13 APP precursors and the final product AAP (Table 1). All matrix-matched calibration curves, prepared separately for precursors and AAP, had a R^2 value of at least 0.99. The range of quantitation was from the lower quantification limit up to 500 μg/L for all the AAP precursors, with the exception of TRP which was quantifiable up to 5000 μg/L, while the range of quantitation of AAP was from 0.05 up to 2 μg/L. The method characteristics linearity, LOD and LOQ, within-run precision (RSD%) and trueness determined for each precursor, are shown in Table 1.

The method was also implemented with a suspect-screening approach that allowed the evaluation of 5 additional compounds (radical cation, FAP, Ox-IAA, IAA-SO_3H and IAA-hexoside). *In-house* prepared standards were used to identify the RT of the different metabolites (radical cation, FAP, Ox-IAA and IAA-SO_3H), and Mass Frontier 8.0 (Thermo Scientific) was used to evaluate the fragmentation profiles (Table 2, Fig. S1 a, b, c, d) to confirm the identity of the detected compounds. With regard to the IAA-hexoside, a peak with an accurate mass of *m/z* 338.1239 (exact mass *m/z* 337.1234, error = −1.5 ppm) was found in wine samples at 5.6 min and showed a loss of a hexose unit

Table 2
Analytical parameters including retention time (RT), normalised collision energy (NCE) and mass error (Δ m/z) of suspect-screening compounds.

Compound	RT (min)	(*m/z*)	Δ *m/z* (ppm)	NCE	Fragments *(m/z)*
[M - H]⁻					
IAA-SO_3H	3.0	254.0129	1.57	35	210.0230, 130.0662
[M + H]⁺					
Ox-IAA	4.8	192.0657	3.12	35	146.0597, 174.0552
IAA-hexoside	5.6	338.1239	−1.5	35	206.0818, 302.1027
Radical cation	6.9	175.0626	2.72	35	130.0658
FAP	7.0	164.0698	3.04	35	146.0601, 100.0245

IAA-hexoside = 3-indolacetic acid-hexoside; IAA-SO_3H = indole-acetic acid-2-sulfonate; Ox-IAA = oxidized indole-acetic acid; FAP = formyl-2-aminoacetophenone.

Table 1
Validation parameters including retention time (RT), linearity, limit of detection (LOD), limit of quantification (LOQ), within-run precision (RSD %) and trueness determined for each precursor and AAP.

Compound	RT (min)	$[M + H]^+$ (*m/z*)	Δ *m/z* (ppm)	NCE	Fragments *(m/z)*	Precision (n = 10; RSD %)	Accuracy (n = 3; %)	LOD (μg/L)	LOQ (μg/L)	Linearity range (μg/L)	R^2
KYN	2.9	209.0913	−3.92	35	94.0651, 192.0655	1.0	64	1.25	4	4–500	0.996
TRP	2.9	205.0965	−3.12	35	146.0594, 188.0968	0.9	94	0.5	1.5	1.5–500	0.9945
TAM	3.4	161.1068	3.35	35	138.0337, 144.0802	0.7	86	0.5	1.5	1.5–500	0.9977
IAA-asp	5.6	291.0965	−3.71	10	130.0646, 134.0450	4.3	77	0.5	1.5	1.5–500	0.9992
IAM	5.9	175.0861	−3.03	35	130.0647, 116.0699	2.5	102	1	1.5	1.5–500	0.9984
ILA	6.4	206.0806	−2.77	35	118.0649, 160.0753	1.7	81	1	3	3–500	0.9965
IAA-ala	6.5	247.1066	−4.37	35	130.0651, 90.0553	2.3	104	1	3	3–500	0.999
IAA	7.0	176.0700	−3.24	35	130.0651, 103.0542	1.1	88	1	1.5	1.5–500	0.9995
TRH	7.0	162.0908	−3.08	35	144.0807, 117.0700	1.0	106	0.5	1.5	1.5–500	0.9978
AAP	7.6	136.0753	−2.65	35	118.0647, 113.9645	2.5	103	0.02	0.06	0.02–2	0.9996
IAN	8.1	157.0757	−2.36	35	130.0652, 117.0536	7.5	106	25	75	75–500	0.9965
IPA	8.3	204.0650	−2.45	35	158.0560, 130.0651	16	–	25	75	75–500	0.9927
me-IAA	8.3	190.0856	−3.63	35	130.0647	2.3	74	1	3	3–500	0.9985
SKA	8.8	132.0805	−2.42	35	86.0966, 117.0570	2.7	87	12.5	38	38–500	0.993

KYN = kynurenine; TRP = tryptophan; TAM = tryptamine; IAA-Asp = IN-(3-indolyacetyl)-DL-aspartic acid; IAM = indole-3-acetamide; ILA = indole-3-lactic acid; IAA-ala = N-(3-indolylacetyl)-L-alanine; IAA = 3-indolacetic acid; TRH = tryptophol; AAP = 2-aminoacetophenone; IAN = indole-3-acetonitrile; IPA = indole-pyruvic acid; me-IAA = methyl-indole-3-acetate; SKA = skatole.

(*m/z* −162.053). Although the fragmentation spectra of the IAA-hexoside did not present the mass of the aglycone ion unit (*m/z* 176.0700), the characteristic fragments of *m/z* 130.065 (Fig. S1e) suggested the recognition of the glycosylated molecule. The absence of the IAA ion unit (*m/z* 176.0700) also occurred in the fragmentation spectra of me-IAA and IAA-ala (Fig. S2) and in the IAA-asp spectra it was only present with a very low abundance (less than 5%). When the fragmentation spectra of the IAA-hexoside were evaluated with Mass Frontier 8.0, the molecule was traced back to a N-glycoside form due to the presence of the fragment *m/z* 206.0818 (Fig. S1e).

3.2. Evaluation of antioxidant effect: in-vitro test

A preliminary experiment was performed to explore the antioxidant effect of the adjuvants on AAP formation and thus the potential protection against ATA (Fig. 1). Despite the limited formation of AAP, tannins in general showed the greatest potential against ATA. The results also suggested a not negligible role of fermentation in AAP formation, as a part of AAP was already present prior to the ageing of the wines. The particular *in-vitro* vinification may have led to increased oxygen exposure and thus preliminary AAP formation, a phenomenon which is normally not evident during wine fermentation. Besides the compounds that provided the highest protection, which are tannins, gallic acid, Asc and hydroxytyrosol, 4-methylcatechol demonstrated a particularly prominent protection during the fermentation. Nevertheless, this wine reached a similar AAP level compared to the wines treated with tannins, resulting from AAP formation during the ageing phase. GSH showed some but less protective activity towards AAP compared to the other adjuvants, despite being a very powerful antioxidant (Kritzinger et al., 2013; Nardin et al., 2020; Pastore et al., 2003; Sonni et al., 2011) and promising for the wine industry since it has been authorized as additive (Oiv-Oeno, 2018).

3.3. Evaluation of the antioxidant effect: microwinery sample preparation

3.3.1. Effect of antioxidant addition in wine's AAP-precursor content

Considering these preliminary results, the experimental plan was extended with a further micro winery study. In this section of the study, only authorized adjuvants were taken into account: a grape skin/seed tannin mix (GrT) and a GaT, considering their wide use in winemaking.

Five musts (PB; PG; Rs1, Rs2; Jn) added with the 5 different antioxidant adjuvants (Asc, GSH, ET, GrT and GaT) and the corresponding controls (Ctr) were fermented in duplicate. The 60 wines were divided in 3 aliquots of which one represented the 'young' wine (T_0) and the other two were put in the oven for the accelerated aging (40 °C; 3 days, T_3; 6 days, T_6). Subsequently, the samples were analysed with HPLC-HRMS.

T_0 samples were used to observe the initial AAP-precursor content in wines and evaluate the differences between the differently treated musts pre-fermentation. Among the different considered precursors present in the young wines, IAN, IPA, SKA, and KYN were never detected above the LOD. However, the most relevant AAP precursor IAA was found to be present with a concentration ranging from 20 to 120 μg/L. The distribution of IAA and the various AAP precursors in wines with different antioxidant treatments is visualized in the box plots (Fig. 2). Compared to the Ctr wine (Friedman's test, p value < 0.05), Asc and GrT treated samples had a significantly lower IAA content, while ET and GaT treated wines resulted in significantly higher concentrations. The IAA concentration upon the GSH treatment was found similar compared to the Ctr and no significant difference was found. Smaller though significant differences were found for the three IAA conjugates, with final concentrations varying from 0.1 μg/L to 0.3 μg/L, 0.2 μg/L to 7.6 μg/L and from below LOD to 0.9 μg/L for IAA-ala, IAA-asp and me-IAA, respectively. Significantly higher concentrations of these three precursors were present in wines treated with GaT compared to the control. Additionally, the ET treatment resulted in increased concentrations of IAA-ala and me-IAA, whereas GSH addition led to lower IAA-asp concentrations compared to the Ctr. These results suggest that GaT does not protect wine from potential ATA and even showed an adverse effect with increased concentrations of IAA and IAA conjugates compared to the Ctr. In contrast, Asc, GrT and GSH showed promising results as these treatments led to a reduced presence of IAA in the wines, even if they maintained a possible reservoir of IAA-conjugate forms.

Significant differences between the different antioxidant treatments were also found for other precursors. In particular, IAM (from LOD to 18 μg/L) and ILA (from 13 μg/L to 202 μg/L) resulted in higher concentrations upon the GaT treatment, when ILA was lower for the GSH treatment compared to the Ctr sample. In samples treated with ET or GaT, TAM (from 0.1 μg/L to 1.8 μg/L) and THR (from 515 μg/L to 9.8 mg/L) were present in significantly higher concentrations, whereas TRP (from 112 μg/L to 6.2 mg/L) showed an evident decrease only upon the GrT treatment. When Asc showed high potential by limiting a further IAA formation, this was not demonstrated for the other precursors.

In all box plots of the precursors, with exception of IAA-asp, different outliers or extreme data points were observed. An important variability

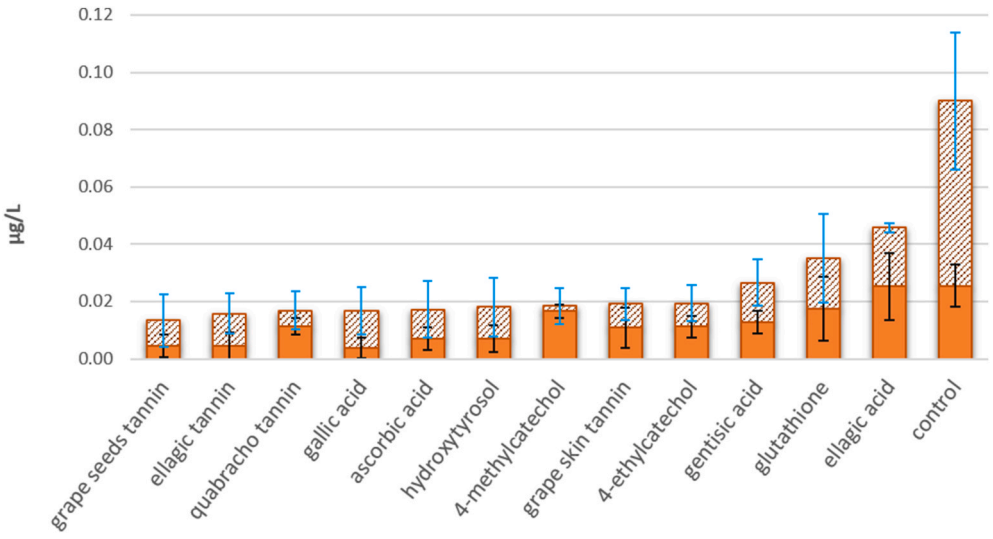

Fig. 1. Formation of 2-aminoacetophenone in wines, produced with different pre-fermentation adjuvant addition, before (full colored) and after an accelerated aging (40 °C; 48 h of heating; striped colour). Represented error bars corresponding to one standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

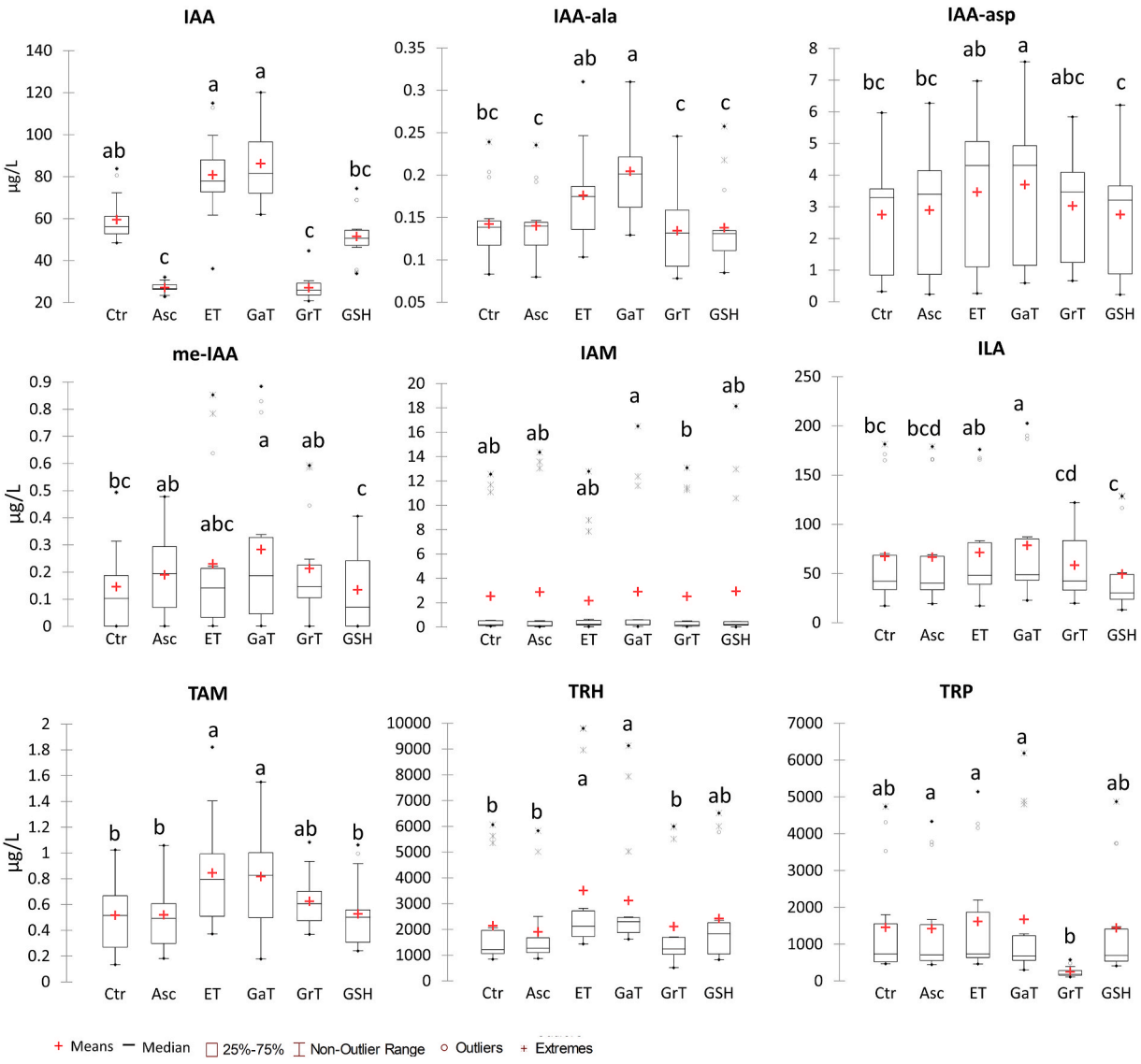

Fig. 2. Box plots of distribution of targeted 2-aminoacetophenone precursors (TAM = tryptamine; IAM = indole-3-acetamide; TRP = tryptophan; TRH = tryptophol; IAA-Asp = IN-(3-indolyacetyl)-DL-aspartic acid; ILA = indole-3-lactic acid; IAA-ala = N-(3-indolylacetyl)-L-alanine; me-IAA = methyl-indole-3-acetate; IAA = 3-indolacetic acid) in wines produced with different treatments (Asc = ascorbic acid, GSH = glutathione, ET = ellagic tannin, GrT = grape tannin, GaT = galla tannin). Different letters (a, b, c) indicate significant differences between the treatments for multiple pairwise comparisons using Nemenyi's procedure (Friedman test, p value < 0.05).

of the precursor concentration was in fact attributed to the different must varieties. PG resulted in a relatively high abundance of ILA, IAM, me-IAA and TRP, in contrast to Rs1, showing increased contents of IAA, IAA-ala and TRH, and Rs2 in TAM. In conclusion, all the treatments demonstrated a different pool of possible ATA precursors, pointing out the antioxidant effects of the different adjuvants. Particularly interesting were the wine samples treated with tannins that resulted in a different final precursor content depending on the botanical origin of the specific tannin.

Regarding the suspect-screening precursor compounds, Fig. 3 shows the distribution of the IAA-SO3H and the IAA-hexoside in the wine treated samples. Results were expressed in area (counts*min) normalised with the total ion current (TIC). Differences were observed in the content of IAA-SO3H depending on the treatments; significantly higher signals were found for the Ctr and GSH wines when lower signals were observed for GaT, GrT and Asc wines. In contrast, the peak signals for the IAA-hexoside were low for all the samples and did not reveal significant differences among adjuvants.

3.3.2. *Effect of antioxidant addition on ATA development*

The effect of the different antioxidant treatments was evaluated by quantifying the AAP content in the artificially aged wines (40 °C; 3 days, T_3; 6 days, T_6). Fig. 4 shows the box plots of the AAP content distribution during the accelerated aging treatment. In all the wines the AAP content increased linearly ($R^2 > 0.9$) during the aging period, reaching the highest values in Jn Ctr (average 3.9 µg/L) and PB Ctr wines (3.5 µg/L). In all the samples protected with the antioxidant treatments the highest AAP content was achieved in Jn wines, except for the Asc treatment of PG. Fig. 4 shows that all antioxidant adjuvants resulted in a decreased rate of AAP formation and a final content that was significantly lower for all treatments with exception of GSH. Although it should be considered that the formation of AAP can continue also after 6 days, the results showed the potential of these antioxidant adjuvants to protect against ATA. In particular, Asc showed the greatest reduction of formation rate with the lowest final concentrations AAP in wine after day 6. The demsar plot (Fig. S3) presents the significant differences between the different treatments for the two aging moments (T_3, a.; T_6, b.). The highest AAP concentrations were found for the Crt wines and were not significantly

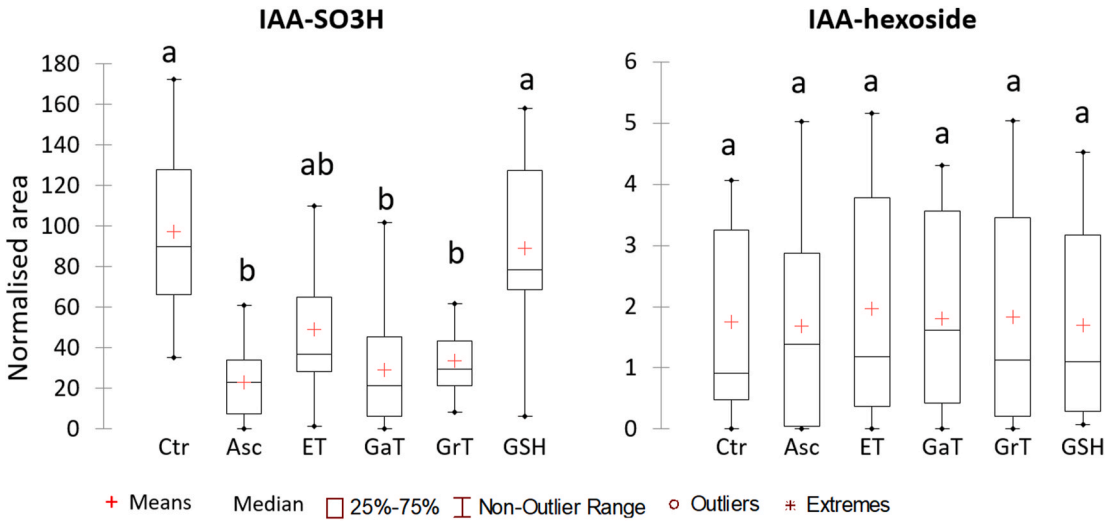

Fig. 3. Box plots of distribution of suspect-screening 2-aminoacetophenone precursors (IAA-SO_3H = indole-acetic acid-2-sulfonate and IAA-hexoside = 3-indolacetic acid-hexoside) in wines produced with different treatments (Asc = ascorbic acid, GSH = glutathione, ET = ellagic tannin, GrT = grape tannin, GaT = galla tannin). Different letters (a, b, c) indicate significant differences between the treatments for multiple pairwise comparisons using Nemenyi's procedure (Friedman test, p value < 0.05).

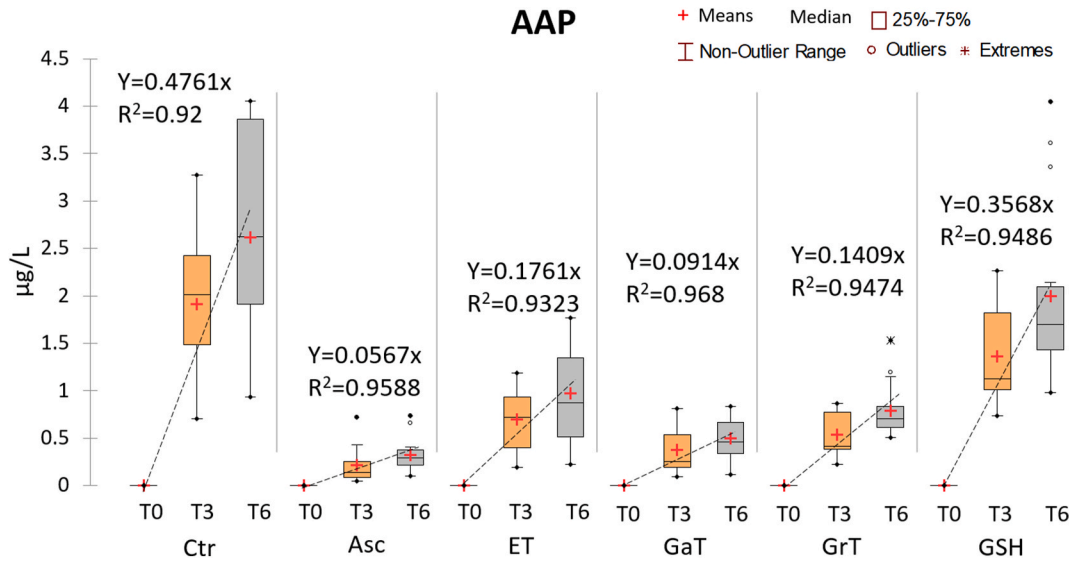

Fig. 4. Box plots of distribution of 2-aminoacetophenone in wines with different treatments (Asc = ascorbic acid, GSH = glutathione, ET = ellagic tannin, GrT = grape tannin, GaT = galla tannin) during the accelerated aging (40 °C; T0 = young wine, T3 = after 3 days heating, T6 = after 6 days heating); — Median trend line (forced through T0).

different from the GSH wines. The AAP content was significantly lower for all the 3 tannin treatments (ET, GaL, GrT) compared to the Ctr, from which the lowest values for GaT and GrT did not significantly differ from the Asc treatment. AAP concentrations at T_6 showed average values of 0.79, 0.5 and 0.32 µg/L and maximum values of 1.5, 0.84 and 0.73 µg/L for GrT, GaT and Asc, respectively. Consequently, Asc, GaT and GrT were designated as the treatments with the highest potential to prevent ATA. Considering Asc, the results confirmed what is reported in the literature (Christoph et al. 1999; ,Schneider, 2016), while for tannins different observations were described (Schneider, 2016). However, the present study involved different tannins and each type of tannin acts differently in preventing the formation of ATA. This could be an explanation why the results were not corresponding to the findings from other researchers.

Comparing these data with the different AAP precursor developments, it is evident that despite the increase of some AAP precursors upon addition of specific antioxidant adjuvants a decrease of AAP in wine after accelerated ageing was observed. GaT for example showed an increased formation of IAA, IAA conjugates and other precursors as compared to the control and other treatments, but resulted in the lowest concentration of AAP among the different adjuvants.

Interestingly, the generally potent antioxidant GSH did not belong to the most effective treatments for this purpose, which was not in line with previous findings in literature (Dubourdieu & Lavigne, 2004). However, differently from what is reported by Dubourdieu and Lavigne (2004) in the present work GSH was added post fermentation. Our observations are more in accordance with those reported by Schneider (2014), describing that the effects were not always significant despite additions of GSH up to 150 mg/L.

3.4. Study of IAA, IAA-conjugates and metabolites evolution during wine aging

To further explore the degradation and formation kinetics of the different compounds involved in AAP formation, different precursors were followed throughout 25 days of accelerated ageing of two different PG wines (in triplicates): with an Asc treatment and without treatment. The trends of formation and/or degradation of IAA and its conjugates, radical cation, FAP and Ox-IAA were evaluated, analysing the samples with the HPLC-HRMS and peak areas of each compound were standardized to 100%. Fig. 5 shows the degradation of IAA and its conjugates during the accelerated aging of an untreated wine (a.) and a wine with added Asc (b.).

In general, the trends of the different precursors showed to be similar for both two different wines. Nevertheless, some differences were found, for example the concentration of the IAA-asp decreased more rapidly for the treated wine (initial concentrations 1 μg/L, concentration after 6 days 0.3 μg/L), compared to the untreated wine (0.8 μg/L, 0.4 μg/L, respectively). Despite that, there was a final stabilization in the Asc treated wine with a final concentration of 0.5 μg/L compared to 0.2 μg/L for the untreated wine. On the contrary, the other IAA conjugated form, IAA-ala, started in both cases with a concentration of 0.2 μg/L which decreased after 25 days to 0.1 μg/L. These two precursors remained above 50% for the Asc-treated wine, suggesting a protective role from this antioxidant by slowing down degradation of these precursors. Among the different precursors, IAA showed the most rapid decrease during the accelerated ageing period, especially for the Asc treated wine. The initial (day 0) IAA contents for both treatments were different, 90 μg/L and 33 μg/L for the untreated wines and Asc-treated wines, respectively, which shows the difference in IAA accumulation resulting from the treatment. After accelerated ageing, the IAA content decreased from 90 to 13 μg/L and from 33 to 3 μg/L, for untreated and Asc-treated wines respectively. This would imply that the absolute reduction of IAA is greater for the untreated wine, but the increased available amount at day 0 should be considered as a possible influencing factor. A completely different trend was observed for IAA-SO_3H, a compound already observed by Hoenicke, Simat, et al. (2002), which showed an increase throughout the accelerated ageing process. This accumulation occurred more rapidly in the untreated wine, reaching a higher maximum signal at day 5 of 1.5×10^5 count*min compared to the Asc-treated wine, which reached its maximum later, at day 15 with 1.9×10^3 count*min. Moreover, a decreasing trend of IAA-SO_3H was only observed for the Asc-treated wine and initiated after reaching the maximum signal. In contrast, the untreated wine maintained a stable value until day 25, however, this IAA-SO_3H pool could potentially be subjected to degradation upon an extended period of accelerated ageing. Also for the IAA-hexoside an initial formation was observed, reaching the maximum more rapidly in the Asc-treated wine (3.7×10^3 count*min) compared to the untreated wine (4×10^3 count*min) and was followed by degradation in both cases. Confirming what reported by Hoenicke, Simat,

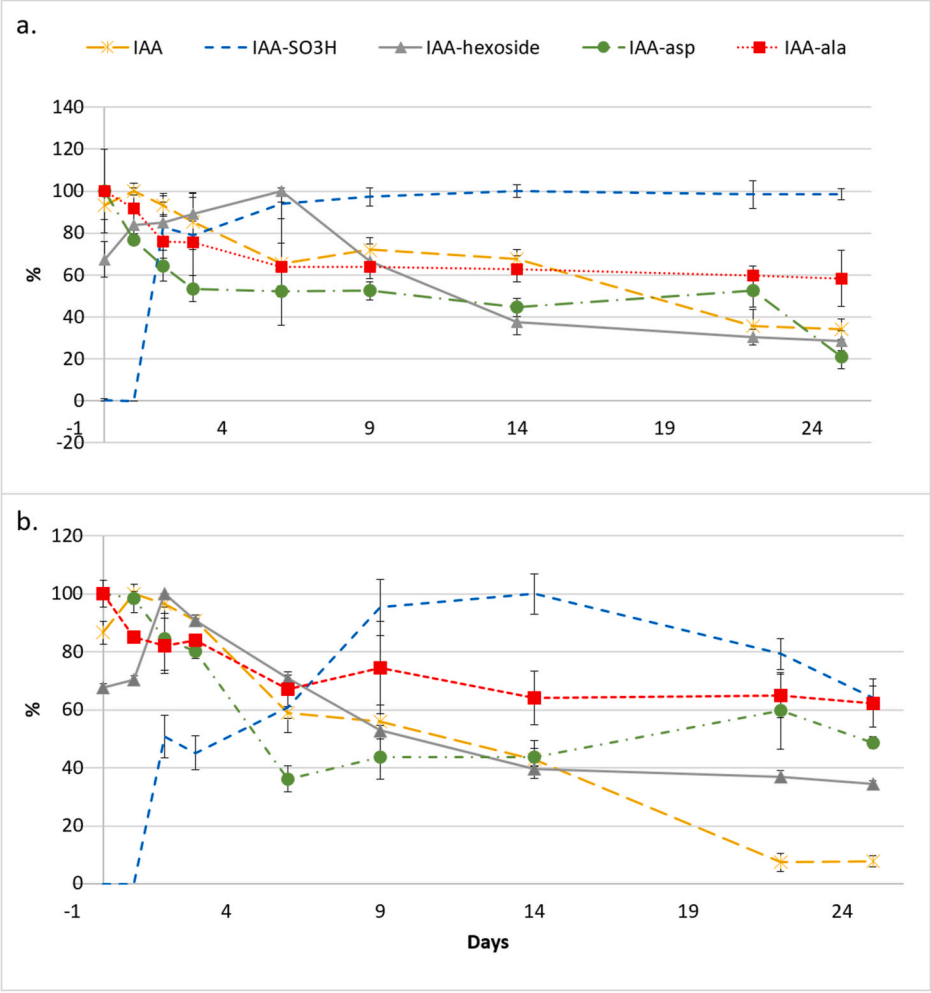

Fig. 5. Graphs of evolution in wine (a) and wine added with 100 mg/L of ascorbic acid (b), of IAA (3-indolacetic acid) and IAA-conjugates (IAA-ala = N-(3-indolylacetyl)-L-alanine; IAA-ala = N-(3-indolylacetyl)-L-alanine; IAA-SO_3H = indole-acetic acid-2-sulfonate; IAA-hexoside = 3-indolacetic acid-hexoside) during an accelerated ageing (25 days, 40 °C). Represented error bars corresponding to one standard deviation.

et al. (2002), Fig. 6 shows the intermediate metabolites of the oxidative reaction (radical cation and FAP) and the final products (AAP and Ox-IAA). The trends of the different compounds appeared to be really similar for both untreated wine (a.) and Asc-treated wine (b.). The first molecule that is produced during the reaction is the radical cation, which increased rapidly in the first days and then decreased as quickly below the detection limit. FAP and Ox-IAA, two metabolites already present in wine before the aging, increased in the first days and then remained constant. The final signals of Ox-IAA were very similar for both the studies: 3.8×10^4 and 3.2×10^4 for no-treated and Asc-treated wines respectively. In contrast, FAP in the Asc-treated wine resulted in a lower final signal and increased less during the 25 days compared to the untreated wine. Finally, AAP showed a constant increase that seems to be continuing at the end of the accelerated ageing period. The trends of this intermediate are similar for both untreated and treated wine, but a clear difference was found regarding the final detected concentration: 6.8 μg/L for the untreated wine and 1.3 μg/L for the Asc-treated wine. This result can be an interesting preliminary indication of the protection ability of Asc against ATA.

In conclusion, the addition of Asc limited both the production of the precursor IAA and the final product AAP which directly involves the defect of ATA. Nevertheless, a reserve of IAA and FAP remained present in the wines after 25 days of accelerated ageing which is considered a possible reservoir of AAP release over time (Katrin Hoenicke, Simat, et al., 2002).

3.5. AAP predictive model

The possible prediction of AAP formation in wines was studied using the ANCOVA linear modelisation, in order to determine the variation of the AAP final content after the accelerating aging (T_6) in relationship to the different grape varieties, the antioxidant treatments (qualitative variables) and the IAA content in young wines (T_0; qualitative variable). Fig. 7 shows the chart of the ANCOVA model representation with the predicted AAP (μg/L) values versus the observed AAP (μg/L) values of each variety (a.) and antioxidant treatment (b.). The different labels can help to understand and confirm the importance of the different adjuvant treatments.

The predicted model had an R^2 (coefficient of determination) of 0.804 indicating that 80.4% of the variability of the dependent variable was explained by the explanatory variables. The goodness of the model was also expressed by the MSE and RMSE, with values of 0.242 and 0.492, respectively. The Fisher's F test resulted in a Fisher valence value less than 0.0001 meaning a risk lower than 0.01% in assuming that the null hypothesis (no effect of the two explanatory variables) is wrong. However, among the three selected variables (grape variety, antioxidant treatment and IAA content), IAA content does not have a significant contribution (0.466 as Pr > F in Type I and III Sum of Squares analysis).

4. Conclusions

In this study we confirmed the effectiveness of several antioxidant

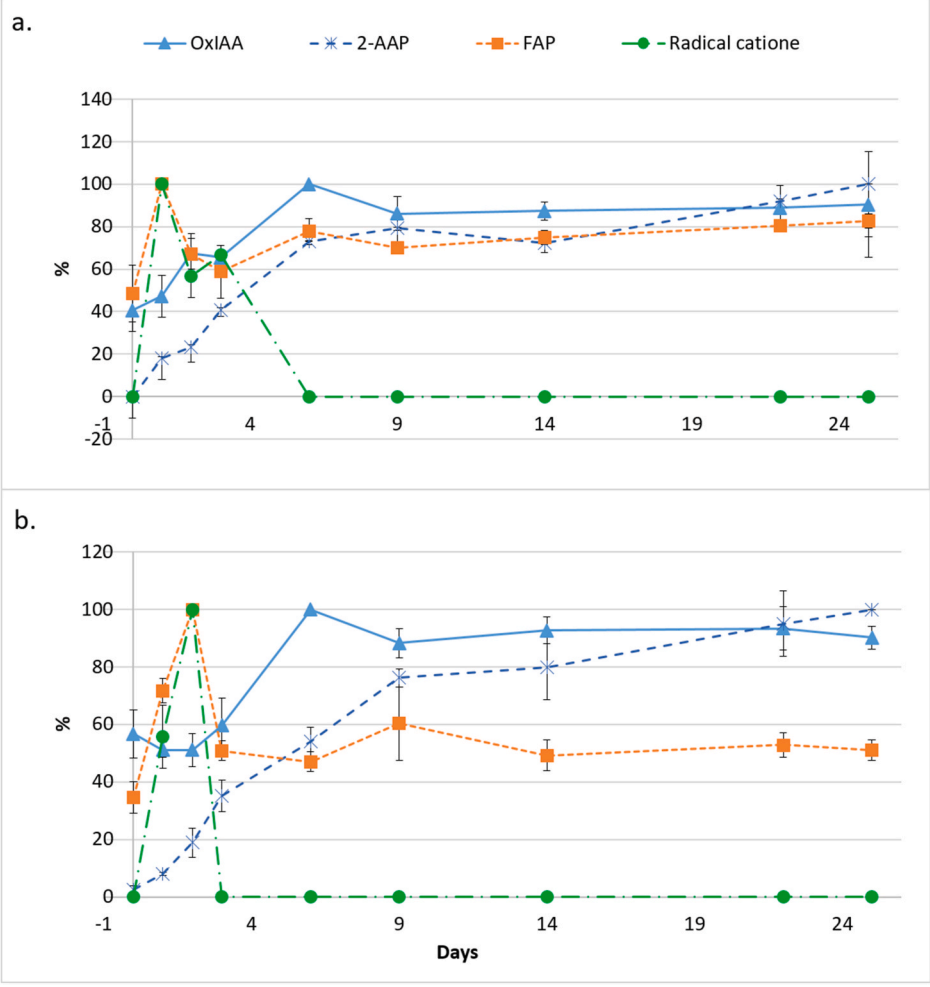

Fig. 6. Graphs of evolution, in wine (a) and wine added with 100 mg/L of ascorbic acid (b), Radical cation, FAP (formyl-2-aminoacetophenone), Ox-IAA (oxidized indole-acetic acid) and AAP (2-aminoacetophenone) during an accelerated ageing (25 days, 40 °C). Represented error bars corresponding to one standard deviation.

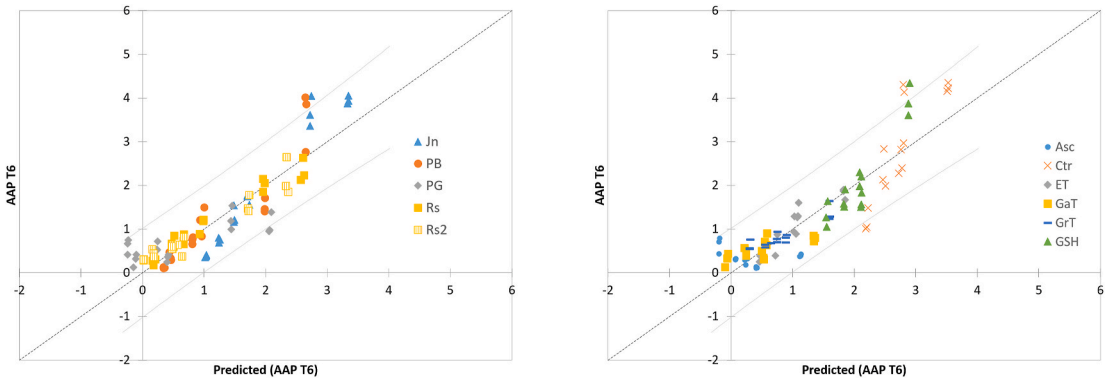

Fig. 7. Chart of the Ancova model representation with the predicted AAP (μg/L) values versus the observed AAP (μg/L) values. Confidence intervals identify potential outliers.

adjuvants against the possible development of ATA, among which the most promising Asc, GrT and GaT. Asc and GrT addition induced a reduced production of the IAA precursors, whereas GaT provided protection particularly during the storing period by preventing the AAP formation despite the IAA content. Finally, an ANCOVA linear modelling, using the grape variety, the IAA content before aging and the antioxidant treatment of the must, showed a promising capability to predict the possible development of the AAP in wine during fining processing. This model could be implemented in future studies by inserting additional grape varieties and evaluating different vintages in order to make it more robust for routine use in ATA possible defect screening.

Declaration of competing interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

CRediT authorship contribution statement

Tiziana Nardin: Methodology, Data curation, Writing – original draft. **Tomas Roman:** Resources, Investigation. **Susanne Dekker:** Writing – original draft. **Giorgio Nicolini:** Investigation. **Francesco Thei:** Formal analysis. **Barbara Masina:** Formal analysis. **Roberto Larcher:** Conceptualization, Supervision.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2021.112639.

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